Nutraceutical Beverages

Chemistry, Nutrition, and Health Effects

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Nutraceutical Beverages

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Preface

Epidemiological evidences have pointed to the link between dietary intake of fruits, vegetables, and products thereof as well as health and disease status. These studies have served as a catalyst for driving the demand for foods and food ingredients or nutraceuticals in order to ameliorate disease and promote health. Beverages derived from fruits and vegetables are important because they serve as a rich source of vitamin C, carotenoids, phenolics, and polyphenolics as well as other bioactives. The mechanism of action of components of these beverages as well as those derived from soy and dairy products such as milk are different, depending on the components of the cocktail of chemicals present. The bioactives in nutraceutical beverages may act synergistically with one another to render their effects and these may be amplified through fortification, cultivating practices, or through biotechnological means. This book presents a state-of-the-art contribution of worldwide researchers in an attempt to unravel different factors involved in the formulation, chemistry, nutrition, and health effects of nutraceutical beverages. The book is intended to serve as a focal point for dissemination of recent developments in the field with consideration of the achievements of the past. The material covered will be of interest to nutritionists, biochemists, and health professionals as well as to consumers and industries.

We are most grateful to the authors for their outstanding contributions and their efforts in the preparation of this book. Their insight and research may lead us to healthier lives.

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Nutraceutical Beverages

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Chapter 1

Nutraceutical Beverages: An Overview

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Nutraceutical beverages originate primarily from fruits and vegetables sources, but also include those from other plants such as tea, coffee, cocoa, soybean as well as animal products like milk and dairy-based and alcoholic drinks. The health benefits of such beverages are rendered via different mechanistic pathways. However, the main criterion for acceptance of such beverages is their taste and flavor quality. Thus, formulation of high quality beverages with good taste, aroma and shelf-life stability is important for their adequate levels of consumption that are needed for health promotion and disease prevention.

Considerable epidemiological evidence has shown that consumption of fruits and vegetables is associated with reduced risk of chronic diseases of aging as well as cardiovascular and several types of cancer (*1,2*). The phytochemicals present in plant foods, including fruits and vegetables, have been shown to be responsible for health benefits of such foods. While the mode of action of phytochemicals present as well as their chemical composition is varied, compounds with antioxidant potentials such as phenolics and polyphenolics appear to be important. The antioxidant activity of vitamin C in many cases is less important than that of phenolics such as flavonoids which are dominant in fruits (*3*).

Recent focus has been on the identification of bioactives in a wide range of foods, including fruits, beverages and related commodities. The mechanism by which bioactives in foods render their beneficial health effects has also been of much interest. These developments have intensified attention to nutraceuticals and functional foods. By definition, nutraceutical is referred to components in food, both nutrient and non-nutrient, that render beneficial health effect through disease prevention (*4*). These are generally used in the medicinal form of pills, capsules, liquids and alike. On the other hand, foods that contain physiologically active ingredients with health benefits above their basic nutrition are known as functional foods and these are in appearance similar to traditional foods.

While anecdotal evidence has provided much of the basic information about health benefits of certain nutraceuticals, scientific basis for efficacy, standardization of products, their stability and safety aspects require adequate attention. Furthermore, metabolism and metabolites of nutraceuticals and bioactives need thorough studies. Such investigations may begin with *in-vitro* and animal studies, but essential proof may require clinical studies. While most consumers may benefit from certain nutraceuticals, some may need to assess risk/benefit of such ingredients based on their genetic background and lifestyle. In addition, development of biomarkers, study of synergistic and antagonistic effects of nutraceuticals and nutraceutical-drug interactions deserve particular attention.

Nutraceuticals and bioactives generally constitute a minor portion of foods in general and of plant foods, in particular. However, through traditional plant breeding and biotechnological means it is possible to amplify the production of bioactives and hence their content or to introduce them in plants in which presence of particular phytochemicals is unusual. Similarly, the content of certain compounds in animal products may be increased. Thus, the content of conjugated linoleic acid and long-chain polyunsaturated fatty acid in milk may be increased through dietary means or by fortification. Bioactive peptides and their content in milk and related products may be amplified. Introduction of certain health beneficial components in foods may also be practiced. Thus, calcium may be added to orange juice to enhance its beneficial health properties. Table I shows the bioactive components of nutraceutical beverages.

Among nutraceutical beverages of interest covered in this publication, beverages based on small fruits and berries, citrus fruit juices, milk and soymilk, caffeinated beverages such as tea, coffee and chocolate, alcoholic beverages such as beer and wines and other fruit juices may be most important. A cursory account of these beverages is provided in subsequent sections of this chapter as well as in other chapters in this book.

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Small fruits

Small fruits of commercial importance generally belong to the *Vaccinium* family and these include blueberries, lowbush berries, rabiteye blueberries, cranberries and partridgeberries in North America and bilberries and lingonberries in Europe. The main active components in these fruits are anthocyanins and other phenolics, mainly flavonoids. The average daily intake of anthocyanins is approximately 200 mg/day in the US (*5*). The antioxidant activity of berries and their respective juices is fairly high as reflected in their ORAC (oxygen radical antioxidant capacity) values (up to 45.9 μmol Trolox equivalents/g of fresh fruit). The content of vitamin C in berries is fairly low and thus it is unimportant to their antioxidant activity (*4*). Small fruits are known to possess antipoliferative effects (*6*) and some, such as cranberries, have

Table I. Examples of nutraceutical beverages and their bioactive components

Beverage	*Bioactive Components*
Berries and small fruits	Anthocyanins, other flavonoids, phenolics, etc.
Citrus fruits	Limonene, auraptene, vitamin C, canthaxanthin, etc.
Milk	Biopeptides (e.g. caseinophosphopeptides), conjugated linoleic acid, etc.
Soy beverages	Isoflavones, other phenolics, etc.
Grapes	Anthocyanins, etc.
Wines and beer	Anthocyanins, oligomeric and polymeric anthocyanitin, etc.
Tea	Catechins, thearubigins, theaflavins, etc.
Coffee	Phenolics, caffeine, etc.
Cocoa/chocolate	Procyanidins, (-)-epicatechin, etc.
Tomato juice	Carotenoids, vitamin C, etc.

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specific role in prevention of other diseases such as urinary tract infection and other disorders (*7,8*). The anthocyanins assume different colors depending on the degree of hydroxylation and methoxylation as well as the pH of the medium. In addition, degree of glycosylation and position of it play a major role in the antioxidant potential of anthocyanins present in berries.

The chemical composition, flavor effects, stability and health benefits of small fruits and their associated beverages are discussed in chapters 2, 4-69 – 8 and 10-12 of this book. These include anticancer activity, cardiovascular protection and protection against urinary tract infection, among others.

Citrus Fruits

Citrus fruits, mainly oranges, lemons, limes, tangerines, tangelos and grapefruits are of commercial significance. The bioactives present in their respective beverages are varied and may include both phenolic and non-phenolic constituents. Limonoids, canthaxanthin (in pink grapefruit) and vitamin C may make a major contribution to their antioxidant potential, in addition to that exerted by their phenolic constituents (*9-11*). Nutraceuticals originating from citrus fruits and their processing by-products are also discussed (see chapters 3, 7 and 9).

Tea, Coffee and Chocolate/Cocoa

Caffeinated beverages of commercial importance include different types of tea (green, black, oolong and puchong) and coffee. Meanwhile, cocoa-based beverages contain mainly theobromine. The phenolics in green tea include catechins, namely (+)-catechin, (-)-epicatechin, (-)-epicatechin gallate, (-)-epigallocatechin and (-)-epigallocatechin gallate. In black tea, condensation products of catechins, namely thearubigins and theaflavins are also present (*12*). Oolong and puchong teas are semi-fermented products and contain both constituents found in green and black teas. Meanwhile, (-)-epicatechin and procyanidins are the main phenolic present in cocoa and chocolate products (*13,14*).

The beneficial effects of tea, coffee and cocoa on health are related to their antioxidant activity which render cardiovascular protection. However, processing of products and their stability characteristics are of much importance to consumers. Thus, flavor aspects of such beverages and their change upon storage is of considerable commercial and scientific interest. Chapters 13 to 19 cover different aspects related to different types of tea, coffee and cocoa.

Soy and Milk

With respect to soy and milk, much has been discussed in the literature about phytoestrogenic effect and other beneficial aspects of soy isoflavones and health benefits of bioactive peptides, such as caseinophosphopeptides, as well as conjugated linoleic acid (*15,16*) in milk (*17*). Fortification of soy with calcium, antioxidants in milk and structural characteristics of soymilk proteins are discussed in chapters 20, 21 and 22 of this book.

Wines and Beer

Alcoholic beverages such as wines and beer have recently been considered as having potential health benefits. This originates from the components available in the starting materials (*18*). In particular, moderate intake of red wine has been associated with cardio protection. The compounds involved have been identified as anthocyanidins and their oligomers/polymers as well as resveratrol (*19*). However, the aroma and taste of wines and beers are important quality characteristics which serve as important factors in their marketing. Chapters 23 to 27 describe different aspects related to the aroma components of wines, role of Maillard reaction products in wine and presence of a natural cooling substance in beer malt.

Isolation, activity, stability and effects

Beverages and foods contain a wide range of bioactives. Many of the bioactives identified may be antioxidative in nature (Chapter 28) or may include bioactive alkoloids (Chapter 29), among others. Beverages and their components may be influenced by a variety of reactions, including Maillard reaction. Products so formed might influence a range of biomolecules such as

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DNA (Chapter 30). Isolation of bioactives for further studies or use as nutraceuticals might also be of interest. Different methods of isolation have been used and explained in the literature. Among technologies available is countercurrent chromatography (*20*), as explained in Chapter 31 of this book. This isolation/purification technique has gained popularity in recent times.

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Chapter 2

Anticancer and Antihypertensive Effects of Small Fruit Juices

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Epidemiological studies indicate that high intakes of fruits and vegetables are associated with a reduced risk of hypertension and cancer. Since only a small part of the flora has been tested for those bioactivities, we chose small fruits as sources of inhibitory activity of angiotensin I-converting enzyme (ACE), differentiation-inducing activity against HL-60 leukemia cells, and antiproliferative activity toward several cancer cell lines. Among 43 juices, prepared from small fruits mainly grown in northern part of Japan, six exhibited ACE inhibition, while four demonstrated potent differentiation-inducing and antiproliferative activities to cancer cell lines, and yet low cytotoxicity toward normal cell lines. The results demonstrated the feasibility of the physiological screening on the small fruits in order to identify plants rich in anti-hypertensive and/or anticancer substances.

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There is an increasing interest in health-promoting agents of plant origin. It was suggested that lifestyle changes in activity (walking etc.) and diet can reduce risk for vascular disease and cancer.

Hypertension is a highly prevalent risk factor for vascular disease, therefore prevention of hypertension is essential for the reduction of cardiovascular morbidity and mortality. Intensive cohort investigation have indicated that a healthy diet of fresh fruits, vegetable and whole grains is beneficial, because it improves the lipid levels and provides high levels of natural antioxidants. Some active components from foods of plant origin have been found as the candidates to lower blood pressure (*1, 2*).

There is also epidemiological evidence that high intakes of fruits and vegetables are associated with a reduced risk of cancer (*3*). The association is generally most marked for epithelial cancer, apparently stronger for those of the digestive and respiratory tracts, and somewhat weaker for hormone-related cancer (*4*). Several plant-derived drugs have been developed in medical oncology for the following reasons. First, administration of fruits to rodents protects against chemical carcinogenesis. Second, many seemingly unrelated compounds including flavonoids, coumarins, cinnamates, and other phenolics, can protect rodents against chemical carcinogens.

Since only a small part of the flora has been tested for any kind of bioactivity, we chose small fruits as sources of anti-hypertensive and anti-cancer activities. We have prepared juices from various small fruit plants, which grow primarily in the northern part of Japan, especially on Hokkaido Island. These juices were subjected to screening for anti-hypertensive activity by inhibition of angiotensin I-converting enzyme (ACE) and anti-cancer activity by HL-60 differentiation-inducing activity.

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Fruit Sample Preparation

All fruits were harvested from trees at the Hokkaido Forestry Research Institute, Bibai, Hokkaido, Japan in July - September 1996. Fruit was homogenized in ethanol and the ethanolic extract was filtrated, concentrated *in vacuo*, and dissolved in distilled water (concentration: 10 mg/mL) for ACE inhibitory assay, and dissolved in RPMI1640 medium (concentration: 10 mg/mL) for anticancer assay.

Anti-Hypertensive Activity

ACE Inhibitory Assay. The procedure was following the method described in the literature (*5*) with minor modification. One unit of ACE from rabbit lung

(Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 8 mL of 125 mM borate buffer (pH 8.3) containing 1 M NaCl. To 50 μL of the enzyme solution was added 50 μL of fruit sample solution (10 mg/mL), and it was incubated for 5 min at 37°C. Synthetic substrate Bz-Gly-His-Leu (Peptide Institute Inc., Osaka, Japan) solution, 3.5 mM in the same buffer, of 150 μM was added to the mixture, then it was further incubated for 20 min. The reaction was stopped by adding 50 μL of 1 M HCl and the mixture was directly injected to HPLC equipped with ODS column [mobile phase: 80% 20 mM phosphate buffer (pH 3.0) in acetonitrile; flow rate: 1 mL/min] , and hippuric acid produced was detected at 254 nm. The inhibition of enzyme activity was calculated by the amount of hippuric acid based on that of negative control experiment where water was added to the enzyme reaction mixture instead of fruit sample.

Anti-Hypertensive Effects. Among 40 small fruit samples, juices of *Elaeagunus multiflora* Thunb. (Japanese name: Natsugumi; IC_{50}: 1.4×10^2 μg/mL), *Elaeagnus umbellate* Thunb. (Japanese name: Akigumi; IC_{50}: 6.9×10^2 μg/mL), *Rubus idaeus* L. (Japanese name; Yoroppa Ki-ichigo; e.g. European raspberry; IC_{50}: 2.3×10^2 μg/mL), *Ribes latifolium* Janczewski (Japanese name: Ezosuguri; IC_{50}: 4.0×10^2 μg/mL), *Ribes nigrum* Linn. (Japanese name: Kurosuguri; IC_{50}: 4.7×10^2 μg/mL), and *Alonia melanocarpa* (Japanese name: Melanocarpa; IC_{50}: 1.2×10^3 μg/mL), as shown in Table I.

To test anti-hypertensive activity, one methods that we could employ was feeding experiment using a spontaneously hypertensive rat (SHR) as a hypertension model. However, such an animal experiment is expensive and time- and labor-consuming. For screening of large number of samples and for activity-guided fractionation of active principles from natural source, *in vitro* ACE inhibitory assay is more preferable than SHR animal experiment.

As to the small fruits judged to active in this study, nothing has been reported on their ACE inhibitory activity and/or anti-hypertensive activity. ACE inhibitory activity has been demonstrated from other plant sources, such as seeds from Acacia plant (*1*) and Ashitaba leaves (*2*), aerial part of *Jasminum* plants (*9*), wheat germ hydrolysate (*10*), fermented soybean (*11*), and soy sauce (*12*). Nicotianamin was found and/or suggested as an active principle in some plants. Although the active plants reported herein do not include those plants reported before, some of them may contain the same active compound. Isolation of active principles from active small fruit juices is undergoing.

Anticancer Activity

Cell differentiation assay. The differentiated HL-60 phenotype is characterized by nitro blue tetrazolium (NBT) reducing, non-specific esterase, specific

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esterase, and phagocytic activities. NBT reduction, non-specific esterase, and phagocytosis is positive in HL-60 cells, which are induced to monocytes/macrophages, whereas HL-60 cells, differentiated to monocyte/granulocytes, increase NBT reducing and specific esterase activities.

Cell proliferation assay. The level of cell proliferation was measured by using alamar Blue (Biosource International, Lewisville, TX, U. S. A.), an oxidation-reduction indicator. The level of proliferation was measured for the cancer and normal cell lines grown in 96-well microtiter plates.

Analysis of Total Phenolics. The total phenolics were determined with Folin-Ciocalteu reagent primarily according to the method described in the literature (*6*, *7*). We used 100 μL of 1/10 or 1/20 diluted sample or 50, 40, 30, 20, 10 mg/L and a 0-blank of standard series of gallic acid solutions plus 500 μL of 1/10 diluted Folin-Ciocalteu stock reagent, followed after 5 min by addition of 400 μL of 7.5% (w/v) Na_2CO_3 solution, and after 2 h at room temperature reading the absorbance at 765 nm. Results were expressed as milligrams of gallic acid equivalent per 10 g of ethanol extract.

Analysis of Total Anthocyanin. The total anthocyanin was estimated by a pH differential method (*8*). Absorbance was measured at 510 nm and 700 nm in buffers at pH1.0 and pH4.5, using $A = (A_{510}\text{-}A_{700})_{\mathrm{pH}1.0} - (A_{510}\text{-}A_{700})_{\mathrm{pH}4.5}$, and a molar extinction coefficient of cyanidin-3-glucoside of 29600. Results were expressed as milligrams of cyanidin 3-glucoside equivalent per 10 g of ethanol extract.

HL-60 differentiation-inducing effects. Among 43 samples, juices of *Actinidia polygama* Maxim. (Japanese name: Matatabi), *Ribes nigrum* (Japanese name: Kurosuguri, the small fruit-producing line), *Rosa rugosa* (Japanese name: Hamanasu), *Rubus parvifolius* (Japanese name: Nawashiroichigo), *Sorbus sambucifolia* M. Roem. (Japanese name: Takanenanakamado), and *Vaccinium smallii* Roem. (Japanese name: Obasunoki) demonstrated potent differentiation-inducing activity towards HL-60, as shown in Table II.

The relationship between NBT-reducing activity and the total phenolics or total anthocyanin content was investigated. A positive correlation was observed between NBT-reducing activity and the total phenolic or total anthocyanin content. The correlation coefficient was much higher between NBT reducing activity and the total phenolics (Figure 1, r = 0.4292) compared to NBT reducing activity and the total anthocyanin (Figure 2, r = 0.1352). Samples, which contained relatively high content of phenolics, namely *A. polygama*, *Rosa rugosa*, *Rubus parvifolius*, *S. sambucifolia*, and *V. smallii*, demonstrated potent HL-60 differentiating activity.

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Table I. ACE Inhibitory Effects of Small Fruit Juices

Plant family	*Scientific Name*	*Japanese Name*	*Extract* (*mg/g fresh fruit*)	*ACE inhibitory activity* [a] (*%*)	*remarks*
Actinidaceae	Actinidia arguta Planch.	Kokuwa	46.5	15	
	Actinidia arguta Planch. cv. Issaikokuwa	Issaikokuwa	17.9	52	
	Actinidia kolomikta Maxim.	Miyamamatatabi	49.1	51	
Caprifoliaceae	Lonicera caerulea var. emphyllocalyx Nakai	Hasukappu	71.4	46	origin; Kiritappu
	Lonicera caerulea var. emphyllocalyx Nakai	Hasukappu	26.6	5	origin; Taiki-cho
	Lonicera caerulea var. emphyllocalyx Nakai	Hasukappu	78.8	25	origin; Tomato, highbush
	Lonicera caerulea var. emphyllocalyx Nakai	Hasukappu	29.6	—	origin; Tomato, lowbush
	Lonicera caerulea var. emphyllocalyx Nakai	Hasukappu	65.3	12	origin; China
	Lonicera caerulea var. edulis Turczaninov	Keyonomi	12.6	29	origin; Taisetsu
	Lonicera caerulea var. edulis Turczaninov	Keyonomi	61.7	59	origin; Yokotsu-dake
	Lonicera caerulea var. edulis Turczaninov	Keyonomi	31.2	3	origin; Bihoro-toge
	Lonicera morrowii A. Gray	Hyoutanboku	63.2	60	
	Viburnum opulus var. calvescens Hara	Kanboku	39.1	6	
	Viburnum wrightii Miquel	Miyamagamazumi	17.7	16	
Elaeagnaceae	Elaeagnus multiflora Thunb.	Natsugumi	88.1	100	
	Elaeagnus umbellata Thunb.	Akigumi	48.7	91	
Ericaceae	Vaccinium praestans Lamb.	Iwatsutsuji	98.5	2	
	Vaccinium smallii A. Gray	Obasunoki	57.2	—	
Hydrangeaceae	Ribes grossularia Linn.	Marusuguri	47.9	—	collected on 7/27/96
	Ribes grossularia Linn.	Marusuguri	115.9	11	collected on 8/8/96
	Ribes japonicum Maxim.	Komagatakesuguri	50.2	—	
	Ribes latifolium Janczewski	Ezosuguri	38.9	100	
	Ribes nigrum Linn.	Kurosuguri	36.9	100	small fruit-producing line
	Ribes nigrum Linn.	Kurosuguri	58.8	17	large fruit-producing line
	Ribes rubrum Linn.	Fusasuguri	28.1	39	

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Lardizabalaceae	Akebia trifoliata Koidz.	Mitsuba-akebi	30.7	3	
Rosaceae	Alonia melanocarpa	Melanocarpa	52.4	76	
	Chaenomeles japonica Lindl.	Kusaboke	27.9	5	
	Cydonia oblonga Miller	Marumero	32.4	—	
	Malus baccata var. mandshurica C. K. Schn.	Ezonokoringo	89.4	—	
	Prunus salicina Linn.	Sumomo	66.9	—	
	Rosa rugosa Thunb.	Hamanasu	44.4	—	
	Rubus idaeus L.	Yoroppa Ki-ichigo	59.2	100	
	Rubus mesogaeus Focke	Kuroichigo	58.6	100	
	Rubus parvifolius Linn.	Nawashiroichigo	60.3	32	
	Rubus phoenicolasius Maxim.	Ebigaraichigo	22.9	5	collected on 8/8/96
	Rubus phoenicolasius Maxim.	Ebigaraichigo	41.4	7	collected on 8/27/96
	Sorbus sambucifolia Roem.	Takanenanakamado	92.1	—	
Schisandraceae	Schisandra chinensis Baill.	Tyosengomishi	169.4	—	
Taxaceae	Taxus baccata L.	Yoroppa Ichii	108.1	6	

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Table II. Anticancer Effects of Small Fruit Juices

Scientific Name	*HL-60 Differentiation*	*Antiproliferative Activity*			
	NBT reducing cell (%)	*A549*	*B16*	*CCRF-HSB-2*	*TGBC11TKB*
Actinidia arguta Planch.	19.3 ± 5.4	> 70	> 70	> 70	> 70
Actinidia arguta Planch. cv. Issaikokuwa	7.3 ± 3.7	> 70	> 70	69.2	> 70
Actinidia Kolomikta Maxim.	49.3 ± 7.4	52.7	39.8	58.1	58.9
Actinidia polygama Maxim.	77.7 ± 5.0	20.5	13.4	8.7	5.9
Akebia trifoliata Koidz.	33.2 ± 9.4	> 70	> 70	> 70	> 70
Alonia melanocarpa	8.3 ± 1.2	> 70	> 70	> 70	> 70
Ampelopsis brevipedunculata Trautv.	45.3 ± 6.7	> 70	> 70	> 70	> 70
Chaenomeles japonica Lindl.	50.8 ± 7.6	> 70	> 70	> 70	66.1
Cydonia oblonga Miller	17.0 ± 7.9	> 70	> 70	> 70	> 70
Elaeagnus multiflora Thunb.	7.0 ± 7.1	> 70	> 70	> 70	> 70
Elaeagnus umbellata Thunb.	10.3 ± 9.9	> 70	> 70	> 70	> 70
Gaultheria Miqueliana Takeda	30.3 ± 5.3	> 70	> 70	> 70	> 70
Lonicera caerulea (Kiritappu)	33.8 ± 5.6	> 70	> 70	> 70	> 70
Lonicera caerulea (Taiki-cho)	24.7 ± 4.2	> 70	> 70	> 70	> 70
Lonicera caerulea (Tomato-tate)	28.8 ± 8.5	> 70	> 70	> 70	> 70
Lonicera caerulea (Tomato-yoko)	23.7 ± 8.2	> 70	> 70	> 70	> 70

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Lonicera caerulea (China)	25.7 ± 8.0	> 70	> 70	> 70	> 70
Lonicera caerulea (Taisetsu)	17.7 ± 0.5	> 70	> 70	> 70	> 70
Lonicera caerulea (Yokotsu-dake)	49.0 ± 0	> 70	> 70	> 70	> 70
Lonicera caerulea (Bihoro-toge)	19.7 ± 3.1	> 70	> 70	> 70	> 70
Lonicera morrowii A. Gray	27.5 ± 9.2	> 70	> 70	> 70	> 70
Malus baccata var. mandshurica C. K. Schn.	10.3 ± 4.9	> 70	> 70	> 70	> 70
Prunus salicina Lindl.	15.2 ± 4.1	> 70	> 70	> 70	> 70
Ribes grossularia L.	21.3 ± 6.5	> 70	> 70	> 70	> 70
Ribes grossularia L.	26.3 ± 2.5	> 70	> 70	> 70	> 70
Ribes idaeus L.	38.3 ± 10.4	> 70	> 70	> 70	> 70
Ribes japonicum Maxim.	10.0 ± 5.9	> 70	> 70	> 70	> 70
Ribes latifolium Jancz.	8.0 ± 2.9	> 70	> 70	> 70	> 70
Ribes nigrum L. (small fruits)	65.0 ± 9.8	> 70	26.5	30.7	22.3
Ribes nigrum L. (large fruits)	9.3 ± 1.7	> 70	> 70	> 70	> 70
Ribes rubrum L.	20.7 ± 9.6	> 70	> 70	> 70	> 70
Rosa rugosa Thunb.	77.7 ± 9.9	49.7	21.2	17.2	12.6
Rubes mesogaeus Focke	7.3 ± 1.2	> 70	> 70	> 70	> 70
Rubus parvifolius L.	64.7 ± 10.8	> 70	65.0	47.6	46.2
Rubus phoenicolasius Maxim.	31.3 ± 6.8	> 70	> 70	> 70	> 70
Rubus phoenicolasius Maxim.	29.5 ± 10.4	> 70	> 70	> 70	> 70

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Table II. *Continued*

Scientific Name	*HL-60 Differentiation*	*Antiproliferative Activity*			
	NBT reducing cell (%)	*A549*	*B16*	*CCRF-HSB-2*	*TGBC11TKB*
Schizandra chinensis Baill.	44.8 ± 7.4	> 70	> 70	> 70	> 70
Sorbus sambucifolia M. Roem.	65.3 ± 8.6	36.7	24.8	15.4	6.6
Taxus baccata L.	14.0 ± 4.3	> 70	> 70	> 70	> 70
Vaccinium smallii A Gray	75.5 ± 5.4	53.5	22.5	14.0	5.2
Vaccinium praestans Lamb.	52.8 ± 5.6	> 70	> 70	> 70	> 70
Viburnum opulus var. sargentii Takeda	52.7 ± 7.0	> 70	68.0	50.2	48.3
Viburnum Wrightii Miq.	16.3 ± 4.6	> 70	> 70	> 70	> 70
Blank	14.5 ± 5.0	100	100	100	100

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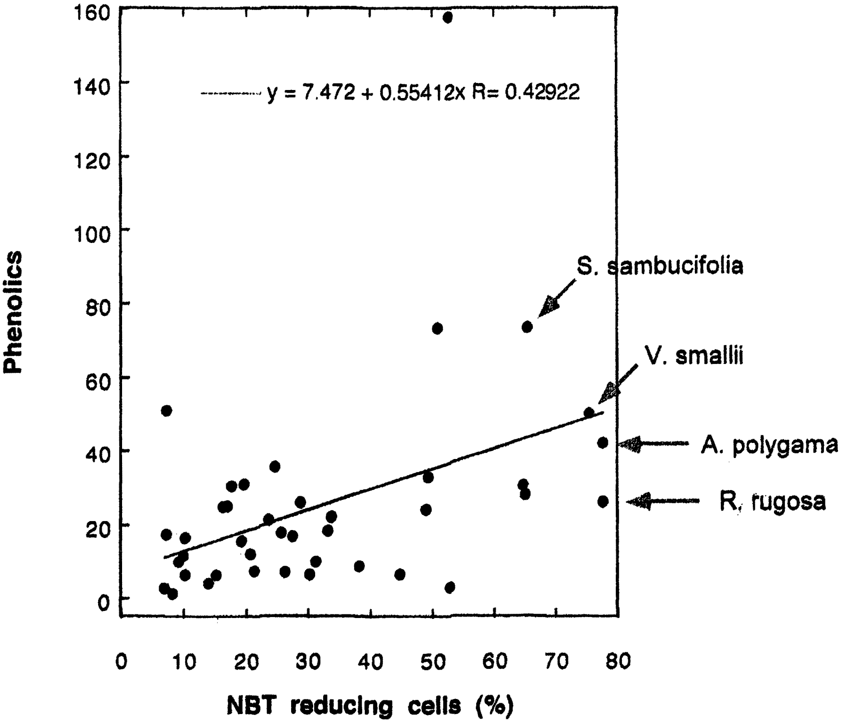

Figure 1. Relation between HL-60 differentiation-inducing activity and amount of total phenolics

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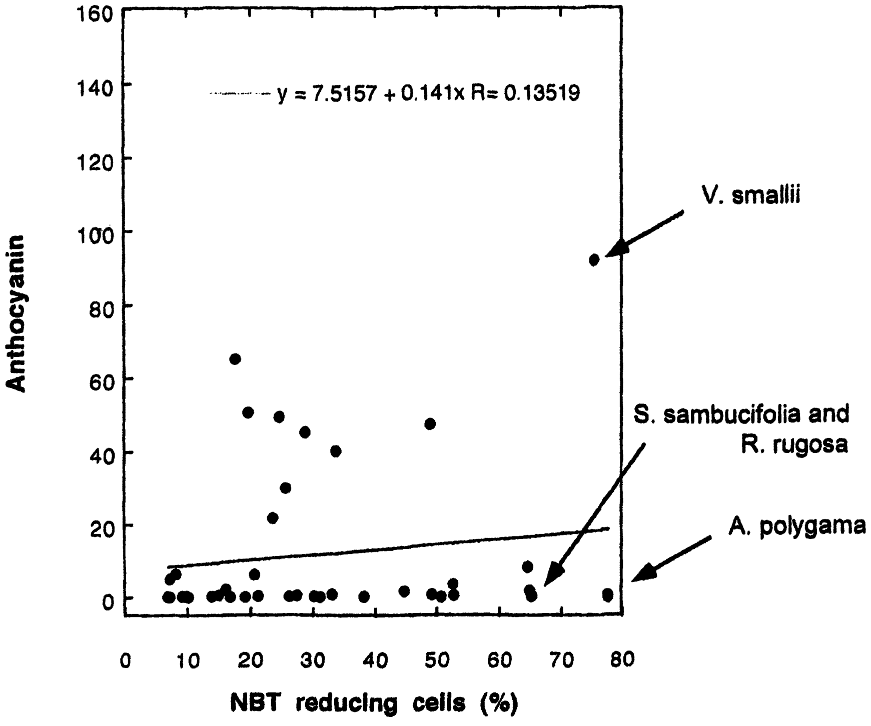

Figure 2. Relation between HL-60 differentiation-inducing activity and amount of total anthocyanin

The results of phenolic assay shows the difference of the total phenolic content between the small-fruit producing and large-fruit producing lines of *Ribes nigrum*, thus suggesting the possible role of phenolics for the NBT reducing activity. On the other hand, the total phenolic or total anthocyanin content, however, could not explain the difference of NBT reducing activity of *Lonicera caerulea* var. emphyllocalyx Nakai (Japanese name: Keyonomi), which had been originally collected at different sites.

Concentration-response effect of *A. polygama*, *Rosa rugosa*, *V. smallii*, and *S. sambucifolia*, which exhibited the potent HL-60 differentiating activity, was examined, and the results are shown in Figure 3. The differentiation-inducing activity was monitored by NBT reducing activity, specific and nonspecific esterase activities, and phagocytic activity, in order to determine the direction of HL-60 cellular differentiation. These juices appeared to induce monocyte/macrophage characteristics, since HL-60 cells treated with these compounds showed NBT reducing activity, non-specific esterase activity, and phagocytic activity in a concentration-dependent manner, whereas they did not express any naphthyl AS-D chloroacetate esterase activity.

Antiproliferative Effects toward several cancer and normal cell lines (14). Antiproliferative effects of the small fruit juices were examined on cellular growth of lung carcinoma (A549), melanin pigment-producing mouse melanoma (B16 melanoma 4A5), T-cell leukemia (CCRF-HSB-2), and gastric cancer cell, and lymph-node metastasized (TGBC11TKB), as shown in Table II. The cellular growth was monitored by reduction of an oxidation-reduction dye, alamar Blue, 3 days after the addition of samples. Among 43 samples examined in this report, *A. polygama* Maxim., *V. smallii* A. Gray, *Ribes nigrum*, *Rosa rugosa* Thunb., and *Sorbus sambucifolia* Roem. demonstrated a potent antiproliferative activity, while a weak activity was found in *Actinidia kolomikta* Maxim. And *Rubus parvifolius* L.

Among the cell lines examined, the growth of TGBC11TKB and CCRF-HSB-2 cells were more sensitive to the small fruit samples and A549 and B16 melanoma 4A5 showed a higher degree of resistance to the antiproliferative activity. The rank order of potency for TGBC11TKB cells was *V. smallii*, *A. polygama*, *S. sambucifolia*, *Rosa rugosa*, and *Ribes nigrum* (the small fruit-producing line).

The antiproliferative activity of *Ribes nigrum* was influenced by the morphological variety of fruits. We have separately examined the fruits from two different lines of *Ribes nigrum*; namely the small fruit-producing line and the large fruit-producing one. There was a marked difference between these fruits. The small fruit inhibited cellular growth of B16 melanoma 4A5, CCRF-HSB-2, and TGBC11TKB approximately 70%, whereas the large fruit did not show any activity.

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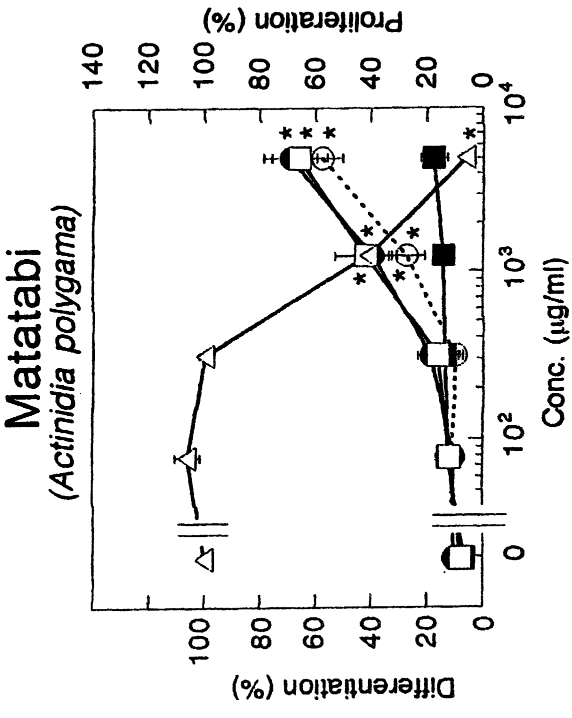

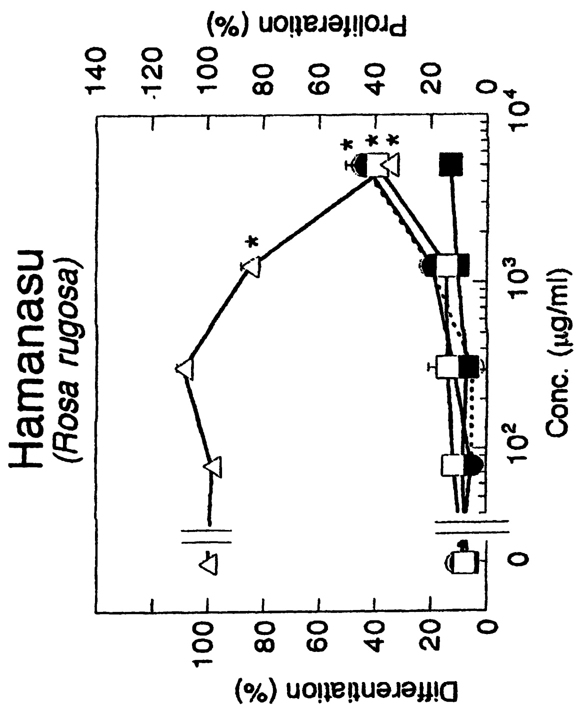

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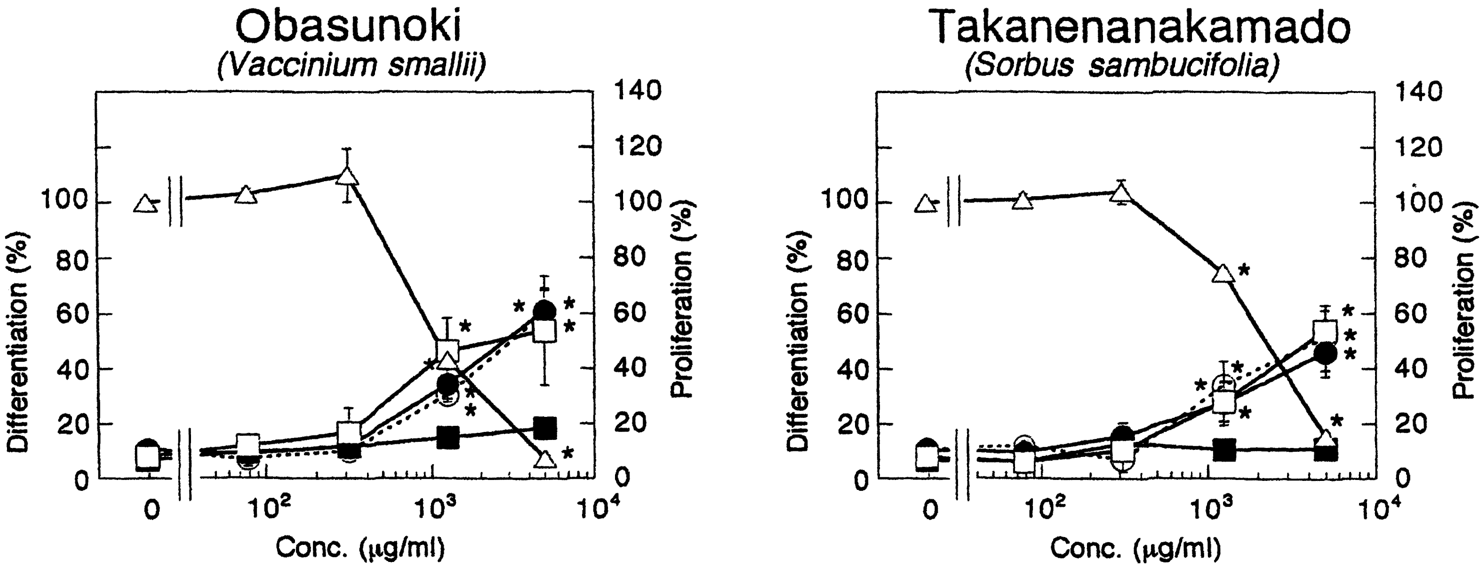

HL-60 proliferation (△), nitro blue tetrazolium reducing activity (●), non-specific esterase activity (□), specific esterase activity(■), phagocytic activity (○).

Figure 3. HL-60 Differentiation-inducing activity

The juices that showed strong activity to cancer cell lines, namely *V. smallii*, *A. polygama*, *Rosa rugosa*, and *S. sambucifolia*, were also examined for their cytotoxicity against normal human cell lines, human foreskin keratinocytes (HFK) and human umbilical vein endothelial (HUVE) cells. These samples, by contrast, are substantially less cytotoxic toward normal human cell lines; the sample did not show any cytotoxicity toward normal human cell lines at a concentration of 5 mg/mL.

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Conclusions

The screening on 43 fruit juices from small fruits growing in northern part of Japan exhibited that anticancer and/or anti-hypertensive biological activity. A massive screening for anti-hypertensive and anti-cancer substances in foods, would lead to discovery and development of new agents with less side-effect.

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Chapter 3

Chemical Composition of Citrus Fruits (Orange, Lemon, and Grapefruit) with Respect to Quality Control of Juice Products

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With expanded consumption and production of citrus fruits and increased demand for healthy nutrition comes the need for knowledge of the chemical constituent of citrus fruits and their products. Thus, the compounds of citrus fruits were analysed. Constituents that are characteristic for citrus species or different fruit parts are useful to determine the authenticity and detect the adulteration in quality control of citrus juices. In expectation of the high profit, citrus juices are subject to falsification because they can be easy manipulated. Many forms of adulteration have been found from simple dilution with water or substitution of cheap ingredients (sugar, acid, colorant essence, and other types of cheap fruit juices) to sophisticated methods as addition of by-products (peel extract or pulp wash) to the juice. Fruit composition is influenced by a large number of natural factors, namely the variety of fruit, the geographical location, the climatic zone, the soil, the degree of maturity as well as other factors. The composition of juice products may be influenced by technology used in processing and packaging.

In spite of all different variables, experiences have shown that a large number of parameters and values are subject to statistical laws. These parameters can be used for the evaluation of juice products with respect to their quality, authenticity and identity, in spite of remarkable variations in their contents.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

It is not possible to establish universally applicable standards for authenticity of processed fruit juices but it is possible to provide minimum or maximum values and/or ranges for individual constituents. The code of practice of Association of Industry of Juices and Nectars (A.I.J.N.) from Juices and Vegetables of the European Union includes reference guidelines, which represent a collection of such minimum and maximum values and/or ranges. These values are used in our laboratory for evaluation the quality of juice products available in the market. Table 1 shows the results of some selected orange juice products purchased from the Hungarian market in 1997, 1999 and 2000.

The presence of high concentration of water soluble pectin, high hesperidin and calcium may indicate the addition of peel extract and pulp wash. The low concentrations of L-malic acid and isocitric acid, and as a consequence, high ratio of citric acid to D-isocitric acid are also signs of manipulation.

As an effect of frequent control, the quality of 100 % orange juice products present in the Hungarian market has improved since 1997 – Out of 12 samples examined, one product had about 50 % fruit content and only one was perfect. In 1999, out of 8 samples two had a pulp wash addition, while in 2000 from 10 samples studied, only one was really manipulated.

The compounds mentioned above should be used together to evaluate the manipulation of citrus juice products. None of these compounds are specific enough to characterise the different part of citrus fruits as juice, albedo or flavedo and the various citrus species. Some early report discussed that there were some differences in protein composition of the exo-, meso- and endocarp of orange, grapefruit, lemon and in species specific immunogenicity of orange and lemon juice. According to these descriptions, proteins seem to be specific enough and particularly relevant to characterise the species and the parts of citrus fruits.

The goal of our program was to develop a new and modern immunochemical method for quality control of commercial citrus juices to detect the manipulation with peel extract.

The experiments consist(ed) of six steps.

1. Identification of juice, albedo, flavedo specific peptides in various citrus species by analytical SDS-PAGE
2. Isolation of these characteristic peptides by preparative gel electrophoresis
3. Development of antibodies against the isolated peptides
4. Analysis of the specificity of isolated peptides on Western blot.
5. Qualitative analysis to detect the peel and juice peptides in citrus juice products by Western blot.
6. Determination of the concentration of characteristic peptides in juice products by ELISA in the near future.

We investigated orange (Citrus sinensis), lemon (Citrus limon), grapefruit (Citrus paradisi).

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Table I. Analysis of 100% orange juices from the market

		1997						*1999*			*2000*		*concentration*		
sample code		*12*	*13*	*14*	*22*	*23*	*24*	*433*	*438*	*440*	*527*	*529*	*range*		
relative density		1,0503	1,0485	1,0497	1,049	1,0438	1,0436	1,0462	1,0469	1,0494	1,0420	1,0420	**min**		**1,045**
glucose	g/l	41	46	37	35	23	31	38	23	39	21	20		**20**	**50**
fructose	g/l	41	44	39	37	24	32	35	25	40	23	22		**20**	**50**
Sucrose	g/l	18	14	25	23	28	20	18	35	25	31	32		**10**	**50**
titer acid pH 8.1	g/l	7,4	7,1	7,3	8,3	6,4	6,1	6,1	7,8	7,0	7,3	6,5		**5,8**	**15,4**
L-malic acid	g/l	0,4	0,4	0,3	0,9	1,2	0,7	0,8	1,3	0,5	1,6	1,6		**0,8**	**3**
citric acid	g/l	8,1	7,6	8,1	8,6	6,9	6,8	6,5	8,4	7,1	7,8	7,1		**6,3**	**17**
D-isocitric acid	mg/l	67	28	68	61	73	67	25	82	47	75	66		**65**	**200**
CS:ICS		122	2662	1192	1422	942	102	262	103	153	105	108	**max**		**130**
Na	mg/l	25	140	23	65	20	94	46	29	172	10	13	**max**		**30**
K	mg/l	1188	744	1220	1286	1540	1241	1228	1945	728	1601	1483		**1300**	**2500**
Ca	mg/l	95	110	95	79	143	177	191	114	161	89	115		**60**	**150**
Mg	mg/l	99	42	96	71	90	90	80	111	68	91	92		**70**	**160**
nitrate	mg/l	14,9	9,8	12,3	2,9	6,9	3,1	8,6	11,6	16	0	9,3	**max**		**5**
phosphate	mg/l	514	256	441	449	520	604	362	548	799	494	531		**353**	**644**
formol number		20	6	20	17	24	20	18	22	17	21	22		**15**	**26**
prolin	mg/l	377	271	339	620	1272	899	1009	863	501	1040	751		**450**	**2090**
water sol. pectin	mg/l	679	643	650	523	734	701	716	583	1509	493	755	**max**		**500**
total carotenoid	mg/l	3,82	4,38	2,9	4,48	1,95	3,06						**max**		**15**
1.fraction	%	34	46	43	6	5,3	25						**max**		**5**
2.fraction	%	4	19	2	15	4	4						**max**		**15**
3.fraction	%	23	1	27	15	12	10								**15**

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hesperidin (D.)	mg/l	475	733	562	741	1506	1484						**max**		**1000**
Brix tabl		12,43	12,01	12,29	12,13	10,89	10,84	11,45	11,62	12,22	10,46	10,46	**min**		**11,2**
soluble solids	g/l	130,6	125,9	129	127,2	113,7	113,2	119,9	121,7	128,2	109	109			
gl:fr		1,00	1,05	0,97	0,96	0,98	0,97	1,08	0,90	0,97	0,91	0,91	**max**		**1**
sugarless solids	g/l	30	21	27	31	37	30	28	37	23	33	34		**24**	**40**
ascorbic acid	mg/l	329	321	321	162	302	112	120	369	83	337	278	**min**		**200**

░ below the range ▓ above the range

Sample preparation. The fruits were peeled. The albedo and flavedo or peel (albedo+flavedo) were separated and cut into small pieces. After peeling the fruits, the juice was obtained by a fruit juice-maker. One gram of peel was extracted in 10 ml water (10 % peel extract). The juice and peel extract were filtered through filter paper. The filtrate was centrifuged at 15,000 xg for 20 min. The sediment was resuspended in Tris-HCl (pH 8.3) buffer and mixed with equal volume of Laemmli's sample buffer. The solution was boiled for 3 min. After centrifugation at 15,000 xg for 10min the supernatant was used for SDS-PAGE.

Gel-electrophoresis. The SDS-PAGE was carried out according to Laemmli (1) on a 13.6% (w/v) separation gel overlaid by 3% stacking gel in a BioRad Mini Gel Chamber. Gels were stained by Coomassie BBR.

Separation of peptides by preparative gel-electrophoresis. Preparative SDS-polyacrylamide gels (1.5mm thick) were used to separate the peptides of the sample and the standard respectively. After electrophoresis, two edges of the gel were cut and stained to determine the position of the peptides of interest, which were then cut out from the gels. Peptides were electroeluted into dialysis bags (Cellulose membrane retainings proteins with MW 12,400 Da) in a Mini Trans Blot chamber (BioRad) for 600 V*h. The peptide samples (~ 6 ml) were dialyzed overnight against distilled water at 4°C and lyophilised. The peptides were redissolved in 60-100 µl of final volume. The purity of peptides was tested by analytical SDS-PAGE and the procedure was repeated again to obtain electrophoretically homogeneous samples.

Preparation of antibodies. The peptide (in 100 µl of PBS with equal volume of Freund's complete adjuvant) was injected into the skin of male BALB/c mice. Animals were boosted 3 weeks later with the same amount of protein. The titer of antibodies was tested in blood samples collected from the tip of tail of the mice. The third booster was normally enough to achieve a useful titer of antibodies against our peptides. After completing the immunizations animals were bled and the IgG fraction was separated from the sera by ammonium sulfate precipitation.

Immunoblotting. Peptides were transferred from SDS-polyacrylamide gel to a sheet of nitrocellulose filter (BioRad, 0.2 µm pore size) by electroblotting according to the method of Towbin *et al.* (2). The nonspecific binding sites were blocked by 5 % Carnation non fat dry milk powder in TBS buffer (0.15 M Tris-HCl, 0.5 M NaCl pH 7.0). After washing three times with TTBS buffer (TBS buffer containing 0.05 % Tween 20) the nitrocellulose sheet was incubated in the presence of the first antibody, diluted in TBS for 12 h at 4 °C. Blot was washed three times in TTBS and incubated with the second antibody (alkaline phosphatase labelled anti-mouse antibody, BioRad) dissolved in TBS in 1:1000 for one hour at room temperature. After the final washes, the blot was developed in a solution of freshly prepared substrate (BCIP and NBT, Bio-Rad). Molecular masses of the positive bands were determined using BioRad prestained standards (Myosin 206 kDa, ß-galactosidase 120 kDa, Bovin serum albumin 84 kDa, Ovalbumin 52 kDa, Carbonic anhydrase 36 kDa, Soybean tripsin inhibitor 30 kDa, Lysozyme 22 kD, Aprotinin 7,5 kDa).

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Juice and peel specific peptides

In citrus fruits, several peptides were identified that occurred exclusively in the juice, or the flavedo or the albedo (3). In orange, seven peptides could be found exclusively in the juice, two of them appearing exclusively in the flavedo. Only in albedo one very pale peptide was detected, which did not appear in the flavedo or in the juice. In grapefruit, seven peptides in the juice and three peptides in the flavedo and one very pale peptide band in the albedo were identified as specific ones, which were not present in the other two parts of the fruit. In lemon juice three, in the albedo one, in the flavedo two specific peptides appeared that did not occur in other parts of the fruit.

The molecular mass of isolated peptides are shown in Table II. The number of peptides isolated by preparative gel electrophoresis were two from orange 2, from three grapefruit juice, one from grapefruit peel and two from lemon juice.

The tissue and species-specific distribution of the antigens have been studied in grapefruit, lemon, and orange by Western blots. Table III shows the distribution of isolated peptides in the juice and peel samples of citrus fruits.

The 27 kDa peptide and the 82 kDa peptide occurred in the juice of all citrus species studied, but did not appear in their peel samples (the antibody developed against 27 kDa peptide isolated from orange juice gave an unspecific cross-reaction with the 29 kDa peptide of the peel sample).

The 65 kDa and the 46 kDa peptides appeared in the peptide samples of grapefruit and lemon juice, but the antibodies did not give a positive reaction with that of orange juice and all citrus peel extracts studied.

Two lemon juice specific peptides with molecular mass of 35 kDa and 28 kDa were isolated. The antibodies gave positive reaction with a single 35 kDa and 28 kDa bands of peptides one by one and did not react with any peptides of orange and grapefruit juices and peel extracts of the three citrus fruits studied.

The 31 kDa peptide isolated from grapefruit peel appeared in all the three peel samples of citrus fruits. This peptide could not be found in grapefruit and lemon juice, but a pale peptide band was detected in orange juice.

Detection of juice and peel peptides in citrus juice products

The next step in this work was to investigate whether the antibodies can differentiate the juice and peel peptides in processed citrus fruits. Figure 1 shows the blots of antibodies developed against 82 kDa citrus juice and 35 kDa lemon juice peptide.

On the blot to the left, the antibody of 82 kDa peptide gave a single positive band with the peptide sample prepared from laboratory-processed grapefruit juice and the commercial grapefruit juice products. One sample

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Table II. Isolation of peptides from citrus fruits

molecular mass	*orange*		*grapefruit*		*lemon*	
of peptide	*juice*	*peel*	*juice*	*peel*	*juice*	*peel*
24 kDa	+					
27 kDa	+					
82 kDa			+			
65 kDa			+			
46 kDa			+			
35 kDa					+	
28 kDa					+	
31 kDa				+		

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Table III. Distribution of isolated peptides in various part of citrus fruits

Polyclonal antibody of peptide	*Dilution of antibody*	*orange*		*grapefruit*		*lemon*	
		juice	*peel*	*juice*	*peel*	*juice*	*peel*
27 kDa	1000-fold	+		+		(+)	
82 kDa	500-fold	(+)		+		+	
65 kDa	250-fold			+		+	
46 kDa	250-fold			+		+	
35 kDa	500-fold					+	
28 kDa	250-fold					+	
31 kDa	15000-fold	(+)	+		+		+

(+) low concentration

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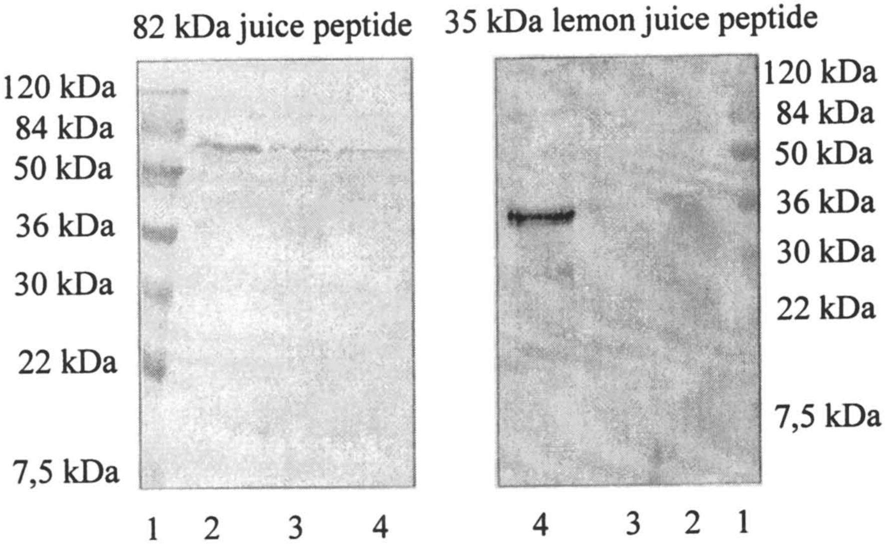
82 kDa juice peptide
35 kDa lemon juice peptide
120 kDa
84 kDa
50 kDa
36 kDa
30 kDa
22 kDa
7,5 kDa
1
2
3
4
120 kDa
84 kDa
50 kDa
36 kDa
30 kDa
22 kDa
7,5 kDa
4
3
2
1

Figure 1. Western blots of antibodies developed against 82 kDa juice peptide and 35 kDa lemon juice peptide. (1) molecular mass standards; (2) grapefruit juice; (3) commercial 40 % grapefruit juice nectar; (4)commercial 100% grapefruit juice; (5) lemon juice; (6) commercial lemon juice concentrate I; (7) commercial lemon juice concentrate II

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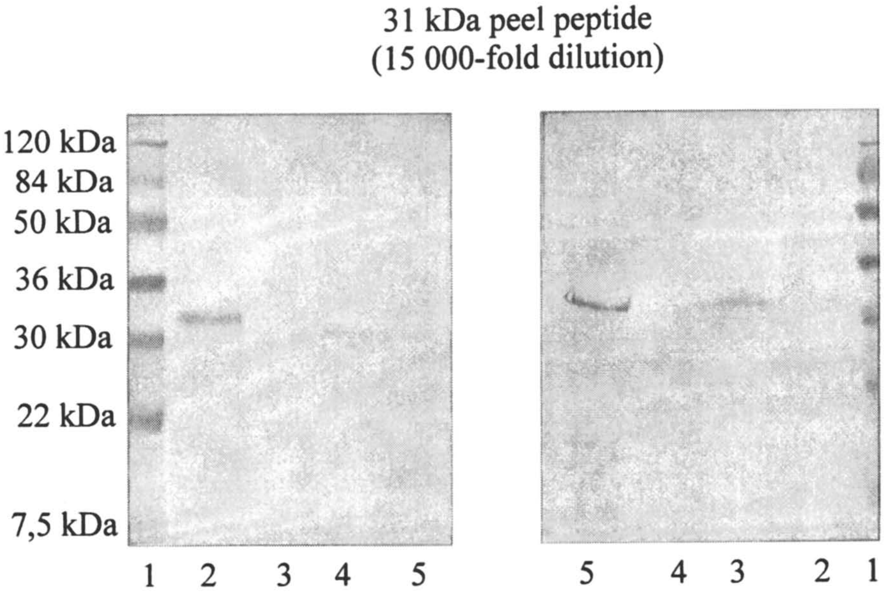

31 kDa peel peptide
(15 000-fold dilution)

Figure 2. Western blots of antibody developed against 31 kDa peel peptide. (1) molecular mass standards; (2) grapefruit peel extract; (3) grapefruit juice; (4) commercial 40 % grapefruit juice nectar; (5) commercial 100% grapefruit juice; (6) lemon peel extract; (7) lemon juice; (8) commercial lemon juice concentrate I; (9) commercial lemon juice concentrate II

was a 100 % grapefruit juice, and the other one was 40 % red grapefruit nectar from the market.

On the blot to the right, the antibody of 35 kDa peptide gave a positive reaction band with laboratory lemon juice and a pale, single positive band with the 35 kDa peptide of commercial lemon juice concentrates. The peptide bands were less intensive in processed lemon juice concentrate than in laboratory-processed lemon juice sample.

Figure 2 shows the immune reaction of the antibody developed against 31 kDa peel peptide. The commercial citrus fruits samples separated on the blots were the same as those in Figure 1.

On the blot to the left, one of the commercial grapefruit juice products declared as 100% grapefruit juice did not give a positive reaction with this antibody while a pale, but well visible band appeared in the peptide sample of the 40% red grapefruit juice nectar. (On the box of the grapefruit nectar it was indicated that the nectar contained fruit flesh as well).

On the blot to the right, a single positive reaction band can be observed with the lemon peel peptides prepared in the laboratory. No cross reactions occurred with the laboratory lemon juice sample. One of the juice concentrates did not give a positive reaction with peel antibody while a pale, but well visible band appeared in the peptide sample of one lemon juice concentrate. The results showed that the antibodies could detect the juice and peel peptides (or peptides originating from by-products) in commercial citrus juice products.

In conclusion the antibody developed against peel peptide can be used to detect the manipulation of commercial citrus juice products by peel extract or other by-products. Antibodies developed against lemon juice peptides can be used to determine the authenticity of lemon juice products. Developing of a quantitative (ELISA) method for determining the juice content and the ratio of possible peel contamination in commercial citrus juice beverages using antibodies raised against these characteristic peptides is intended.

Acknowledgement

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Chapter 4

Cranberry Phytochemicals and Their Health Benefits

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Folklore has long supported the role of cranberry juice in maintaining urinary tract health. Research focused on confirming and understanding how the cranberry provides such a benefit has resulted in considerable scientific evidence supporting this and other potential health benefits. Bioassay directed fractionation identified cranberry proanthocyanidins as the phytochemicals responsible for preventing the adhesion of certain types of *E. coli* associated with urinary tract infections to uroepithelial cells. This unique functionality is also being demonstrated *in vitro* to prevent the adhesion of *H. pylori,* associated with the incidence of certain stomach ulcers, and of certain types of bacteria found in the mouth, associated with dental plaque and gingivitis. Cranberry proanthocyanidins are also potential antioxidants, having significant effect in preventing the *in vitro* oxidation of human LDL. Consumption of cranberry juice powder has also resulted in the *in vivo* reduction of total and LDL cholesterol in an animal model. The proanthocyanidin composition of the cranberry and other phytochemicals of potential health significance, including the anthocyanins, flavonols and phenolic acids are provided.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Cranberries are the small, red fruit of the perennial *Vaccinium macrocarpon*. The fruits are typically 1-2 centimeters in diameter, weigh 1-2 grams, and are known for their bright crimson red color. *Vaccinium* is classified under the Ericaceae family. Other *Vaccinium* species include the high bush, cultivated blueberry (*V. corymbosum*), the low bush, wild blueberry (*V. augustifolium*), the European or small cranberry (*V. oxycoccus*), the bilberry (*V. myrtillus*) and the ligonberry (*V. vitis idaea*). Cranberries are one of the few domesticated berries of significant commercial value that are indigenous to North America, along with blueberry and the fox grape (*Vitis labrusca*), known for the Concord grape cultivar. The name cranberry evolved from craneberry, which originated with colonists who thought the shape of the plant's flower resembled the neck, head and bill of a crane (*1*).

While there are many cultivars of cranberries, commercially nine cultivars predominate and just two, Early Black and Stevens, account for the majority of acreage in production. Early Black is a native selection dating back to the 1857 and Stevens is a hybrid introduced by the United States Department of Agriculture (USDA) in 1950 (*1*). The worldwide production of cranberries totaled 735 million pounds in 1999, of which 85% is grown in the United States and 15% in Canada, while a small amount of fruit is grown in Chile (*2*).

The cranberry industry represents a $2 billion worldwide market (2001 estimate) whose products include fresh fruit, juices, sauces, dried fruit and ingredients, such as frozen fruit, juice concentrates and spray dried powders. Cranberry juice cocktail and cranberry sauce are among the more familiar products in the United States, especially at Thanksgiving.

Macro Composition

The proximate composition of single strength cranberry juice at 7.5 °Brix is listed in Table I (*3*). °Brix is a measure of juice concentration or percent soluble solids, which is technically based on sucrose and is measured using hydrometry or refractometry. °Brix of 7.5 is the accepted industry standard for single strength cranberry juice. As can be seen from Table 1, cranberry juice is almost entirely water and carbohydrates. The 6.9 g of total carbohydrates are comprised of 3.7 g of sugars (Table II), 3.1 g of organic acids (Table III) and 0.1 g dietary fiber. Cranberry juice is uncharacteristic of many other fruit juices where the ratio of fructose to glucose is usually greater than one. In cranberry juice the ratio of fructose to glucose is less than one. Cranberry juice is also uncharacteristic of other fruit juices in the presence and amount of quinic acid that it contains. Quinic acid can be measured and used to determine the percent cranberry juice in a product and for detecting cranberry juice adulteration (*4*). The amount of galacturonic acid in cranberry juice may vary as a result of fruit or juice depectinization. Not listed in Table III are 2-Furoic acid and Oxalic acid. 2-Furoic acid was measured in cranberry juice cocktail as part of the phenolic acid analysis reported later on in this chapter and found at a level of 2.9 ppm. The oxalic acid content in cranberry juice has been reported to be 5 ppm

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(*5*). Oxalic acid, and cranberry juice by association, has been implicated in the formation of kidney stones. However, research has shown the level of oxalic acid in cranberry juice is insufficient for the formation of kidney stones (*6*). The minerals in 100 g cranberry juice include sodium (4 mg), potassium (85 mg), calcium (7 mg) and iron (0.3 mg).

Table I. Proximate Composition of 7.5 °Brix Juice (100 g)

Water	92.9 %
Solids	7.1 %
Calories	27
Total Carbohydrates	6.9 g
Protein	< 0.1 g
Fat	< 0.1 g
Minerals	96 mg
Vitamin C	2 mg

Table II. Cranberry Juice Sugars (%)

Glucose	2.8
Fructose	0.8
Sucrose	< 0.05

Table III. Cranberry Juice Acids (%)

Citric	1.06
Quinic	1.05
Malic	0.78
Galacturonic	0.19
Shikimic	0.02

Cranberry Juice Cocktail

Compared to other fruit juices it can be seen from the macro composition data presented that cranberry juice is relatively low in sugar and high in acid.

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This impacts the perceived sweetness and palatability of cranberry juice. This quality attribute is often described by the ratio of brix to titratable acidity (BAR). For fruit juices normally consumed undiluted and unsweetened, such as apple and grape, this ratio can be in the range of 22 to 24. By comparison, cranberry juice has a BAR of about 3.8, more in line with lemon juice, which has a BAR of 1.6. Beverage manufacturers will typically compensate for the low sugar and high acid content of cranberry juice by blending it with water and sweetener to produce a cranberry juice cocktail (CJC), with a BAR of about 22. In fact, the USDA has established a commercial item description (CID) for this commercial form of cranberry juice, specifying CJC as a beverage that contains at least 25% cranberry juice and 0.26 % quinic acid.

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Cranberry Health Benefits

Cranberries can be thought of as one of the earliest functional foods. In addition to the cranberry's early food uses in such items as pemmican (a dried meat and berry mixture) and sauces, it was used for its medicinal benefits. Native New Englanders used cranberries in poultices for treating wounds and blood poisoning, and for treating urinary disorders, diarrhea and diabetes. Cranberries were also consumed on long ocean voyages to help relieve passengers and crew of the debilitating symptoms of scurvy (*1,7*).

Today, cranberries are known to contain many biologically active components. Currently, between the University of Illinois NAPRALERT database (*8*) and Dr. Duke's Phytochemical and Ethnobotanical databases (*9*), 120 compounds with over 700 biological activities have been reported for *Vaccinium* species in the literature. For instance, there are 40 compounds with 130 effects associated with anticancer activity, 35 compounds with 108 effects associated with antioxidant activity and 25 compounds with 45 effects associated with anti-inflammatory activity. Only a few of these references are specific to *V. macrocarpon*, but this is probably due more to a lack of research with the species, than to a lack of biologically active compounds. It may be assumed that within the *Vaccinium* genus similar biological activities exist in most cases and to varying degrees between the species, as for bacterial anti-adhesion bioactivity found in both cranberry and blueberry (*10*).

Urinary Tract Health

Probably the most reported medicinal use of cranberry juice has been for the "treatment" of urinary tract (urethra, bladder, kidneys and prostate) infections caused by pathogenic bacteria, primarily of the *Escherichia coli* (*E.*

coli) type. Documented research into this benefit dates back to 1923 when scientists examined the hypothesis that due to the low pH and high acid content of cranberry juice, the symptoms and effects of infection were minimized by acidification of the urine (*11*). More recent research is pointing to another mechanism. In 1984 researchers showed that cranberry juice had bacterial anti-adhesion activity, preventing the adhesion of certain *E. coli* to the uroepithelial cells of the urinary tract wall (*12*). This work implied that cranberry juice might have more of a preventative effect than a curative effect, thus helping maintain urinary tract health (UTH). A 1994 clinical study demonstrated the effect of drinking low calorie CJC in a randomized, double blind, placebo controlled study of 153 elderly women for 6 months (*13*). Drinking 10 ounces a day of the CJC reduced the bacteria and white blood cell counts in urine, both markers of urinary tract infection. The test group was half as likely to have high counts and a quarter as likely to continue having high counts. The researchers also confirmed in an *in vitro* anti-adhesion bioassay that the CJC was active while the placebo was not and that the urine of the test group was actually less acidic, dispelling the idea that urinary acidification was responsible for the effect. In 1998 researchers using bioassay directed fractionation identified the proanthocyanidins (PACs), or condensed tannins, in cranberry as being responsible for the anti-adhesion effect (*10*). In 2001 researchers conducting an *in vivo* study demonstrated that the urine of mice fed either CJC or an aqueous solution of purified cranberry PAC extract exhibited anti-adhesion activity, while the urine of mice fed water did not (*14*). This work provides the most conclusive *in vivo* evidence to date linking the PACs from cranberry juice with bacterial anti-adhesion.

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Bacterial Anti-adhesion

Bacteria use fimbriae and pili, proteinaceous surface structures, to adhere to other surfaces. These structures are considered an inherited trait (*15*) and bacteria can express different types of adhesions depending upon the cultural condition (*16*). Cranberry PACs prevent the adhesion of pathogenic *E. coli* bacteria with P-type fimbriae to uroepithelial cell tissue. *E. coli* is responsible for 85% of symptomatic urinary tract infections (UTIs) (*7*). There is also some preliminary research suggesting cranberry PACs may prevent the adhesion of *Helicobacter pylori* to stomach epithelial cells. *H. pylori* causes more than 90% of duodenal ulcers and up to 80% of gastric ulcers (*5*). There is further evidence that cranberry PACs may prevent the adhesion of certain strains of bacteria responsible for dental plaque and gingivitis in the oral cavity (*17*).

Anti-adhesion activity can be measured *in vitro* using a red blood cell hemagglutination bioassay. A more specific agglutination assay can also be conducted *in vitro* using isolated adhesion receptors bonded to resin beads (*18*).

Cardiovascular Health

Preliminary research suggests that cranberries may play a beneficial role in maintaining cardiovascular health. Recently, familial hypocholesterolemic (FH) swine fed a diet containing cranberry juice powder exhibited a significantly lower total and LDL cholesterol level from baseline (*19*). Following a two week washout the FH swine were fed the cranberry powder for four weeks. The analysis of blood samples taken weekly showed an overall decrease of 20 percent in total cholesterol and 22 percent in LDL cholesterol. There was no change in HDL cholesterol. Other work has shown that whole cranberry extracts inhibit LDL oxidation (*20*). In addition, when cranberry flavonoids were fractioned on Sephadex LH-20 and tested for their antioxidant activity, those fractions containing oligomeric and polymeric PACs were found to significantly delay the onset of Cu^{2+} induced human LDL oxidation *in vitro* (*21*). No LDL oxidation was seen at up to 250 min for the oligomeric and polymeric fractions, while the control and earlier eluting fractions containing anthocyanins and flavonols reached maximum oxidation at about 150 min. As this research is still in the early stage, more work is needed to determine the significance of these findings to human health.

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Proanthocyanidins

Proanthocyanidins are polymers of flavan-3-ols linked together by either a single (B-type) or a double (A-type) interflavan bond. The PACs identified in cranberry so far are mostly oligomers and polymers of epicatechin and epigallocatechin containing one or more A-type interflavan bonds (*21-23*). The number of A-type bonds appears to increase as the degree of polymerization increases (*21*). A PAC tetramer is depicted in Figure 1, showing 4-6 and 4-8 B-type single interflavan bonds and a 4-8, 2-O-7 A-type double interflavan bond. There appears to be little or no anti-adhesion activity associated with cranberry PAC monomers, dimers, and higher oligomers with all B-type interflavan bonds (*23*). The A-type interflavan bond appears necessary for PACs to have anti-adhesion activity against P-type fimbriated *E. coli.*

The quantification of PACs poses many analytical challenges due to their polymeric nature and the lack of analytical standards. Structural heterogeneity based on differing monomer units, varying interflavan bond configurations and range of degree of polymerization, complicate the isolation and quantification of individual compounds. Gravimetric assays have been used for quantifying total PAC content by isolating the PAC fraction using C18 reverse phase and Sephadex LH-20 adsorption chromatography (*24*) or Sephadex LH-20 adsorption chromatography followed by precipitation with trivalent ytterbium

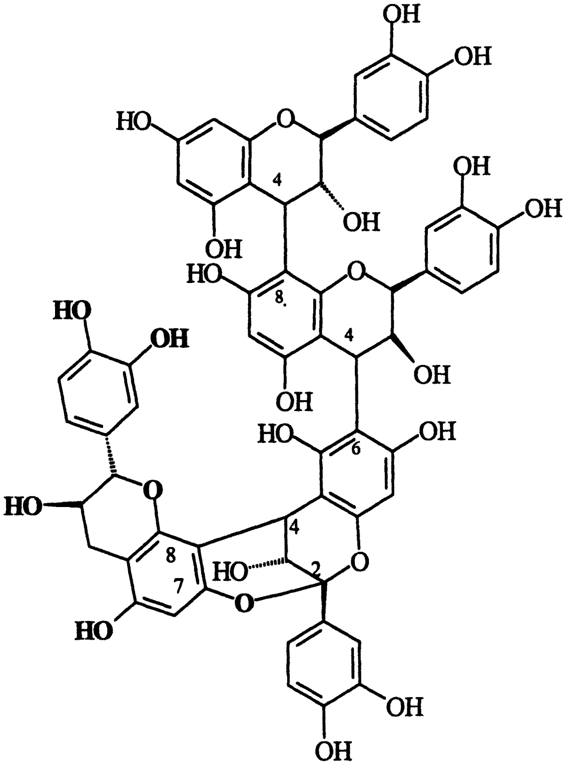

Figure 1. Proanthocyanidin (PAC) Tetramer.

(25). Gravimetric procedures can take two or more days to complete and the amount of sample fractionation scale can limit method sensitivity. Sephadex LH-20 adsorption chromatography combined with a colorimetric assay can improve both method selectivity and sensitivity. Vanillin and 4-dimethylaminocinnimaldehyde (DMAC) are two reagents that have been used for the colorimetric determination of PACs. DMAC is more appropriate for analyzing cranberry samples due to reduced interference from anthocyanins and ascorbic acid. Ideally, the DMAC method should be standardized against a stable sample of the same type, for which the total PAC content has been determined by the gravimetric procedure *(26).* Other colorimetric procedures reported in the literature include Folin Ciocalteu, n-butanol hydrochloride and Prussian blue.

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Table IV lists the PAC content for three common cranberry products determined using the DMAC colorimetric assay, calibrated against 90-MX, a spray-dried cranberry juice concentrate powder. Twelve samples each of cranberry juice cocktail and sweetened dried cranberries were analyzed, with coefficients of variation of 14.7 and 14.8 percent, respectively. The eight samples of whole berry sauce analyzed had a coefficient of variation of 60 percent, with the high variation most likely due to the non-homogeneity of the product. Each of these products provides a similar quantity of PACs on a per serving basis. However, since both gravimetric and colorimetric methods are unable to provide information on the distribution and amount of individual PACs present, this data does not provide any indication of the diversity of cranberry PACs.

Table IV. Proanthocyanidin Content of Cranberry Products per serving size

Cranberry Juice Cocktail	30 mg/48 g mL
Whole Berry Sauce	24 mg/70g
Sweetened Dried Cranberries	32 mg/40g

Acid-catalyzed degradation of PACs in the presence of nucleophiles such as benzylmercaptan (thiolysis) provides information on the monomer composition and possibly the mean degree of polymerization of PACs. When combined with fractionation it can also provide information on PAC distribution (*27*). However, the A-type interflavan bond can not be cleaved to yield the monomers under these conditions (*28*), which for cranberry and other A-type PAC containing plants can result in erroneous conclusions about the monomer composition and mean degree of polymerization.

Chromatographic methods can be used to separate and quantify PAC monomers, oligomers and small polymers, with normal phase (NP) HPLC providing better separation than RP-HPLC (*29*). However, even with the NP-HPLC method it becomes increasingly difficult to resolve PACs greater than dodecamers and as the diversity of the PAC composition increases, the isomers within a degree of polymerization. Further, there are no commercially available PAC analytical standards available other than for the monomers and a few dimers. PAC oligomer and polymer standards are being isolated from natural sources and their purity is limited by the ability to resolve individual compounds.

Finally, mass spectrometric techniques can also be used to separate PACs based on their mass to charge ratio. Both electrospray (ES) mass spectrometry (MS) and matrix assisted laser desorption time of flight (MALDI-TOF) MS can

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be used to profile PACs (*26*). Figure 2 displays the mass spectrum of a cranberry PAC extract purified from 90MX powder using the C18 and Sephadex LH-20 gravimetric fractionation procedure. The extract was re-dissolved in methanol and mixed 3:1 with 10mM ammonium acetate and the solution infused at 5μL per min into the ES/MS source. The mass spectrum is the summation of 100 individual scans from 250-1800 AMU acquired at 3 sec per scan.

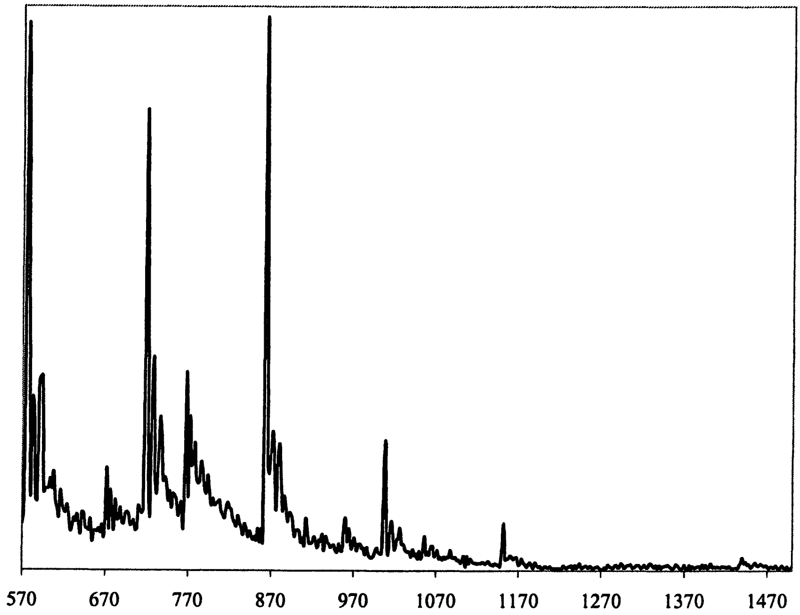

Figure 2. Proanthocyanidin Profile of Cranberry.

This mass spectral profile shows both the type and distribution of individual PACs in cranberries. The multiple charging effect needs to be taken into account when interpreting ES/MS spectra, where a doubly charged tetramer will appear as the mass of a singly charged dimer. This can be recognized by the fact that mass fragment and functional group differences in the spectra will be similarly affected. MALDI-TOF MS does not face this problem and also provides a high degree of mass resolution, which can be useful in accurately identifying PACs with A-type interflavan bonds and other PAC compositional differences.

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Anthocyanins

The anthocyanins are a key cranberry quality attribute for the color they impart to the fruit and juice. The basic anthocyanidin structure is shown in Figure 3. Different forms arise from hydroxy and methoxy substitutions at positions 3' and 5'. Anthocyanins are the result of glycosylation of the anthocyanidin aglycone, at position 3 in the case of cranberry anthocyanins.

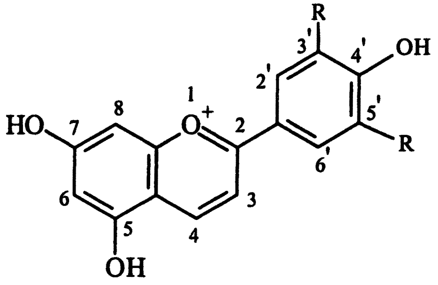

Figure 3. Anthocyanidin Aglycone.

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The anthocyanins are reported to have a number of biological activities. These include antioxidant, antibacterial, anti-fungal and anti-mutagenic activity. They may also have an impact on cardiovascular health through improved capillary fragility. The anthocyanoside fraction of *V. myrtillus* extracts was attributed to improved function of the ophthalmic and vascular systems, after it was noticed during the second world war that pilots eating bilberry jams had better twilight vision (*30*).

The data presented in Table V were obtained for 12 samples of cranberry juice cocktail, three each from four different bottling plants, using a reverse phase (RP) HPLC method with diode array detection (DAD). Chromatography was done on a C18 column using a binary gradient of 2% acetic acid and 20:80 (v/v) 2% acetic acid:acetonitrile. Cyanidin-3-galactoside was used as an external standard to quantify all six anthocyanins. The results are reported on an as tested basis. The coefficient of variation for the results ranged from 40 to 50%.

From the data presented in Table V it can be seen that the total anthocyanin content was about 8 mg/kg for this set of CJC samples. By comparison, a total anthocyanin content of 12-15 mg/kg has also been reported for cranberry juice cocktail (*31*) and we have seen values as high as 25 mg/kg in other samples of cranberry juice cocktail (unpublished data). While there may be variability in the total anthocyanin content of cranberry juice, due to such factors as year-to-year crop variation, it should be noted that the anthocyanin profile and ratio of

Table V. Anthocyanins of Cranberry Juice Cocktail (mg/L)

Cyanidin-3-galactoside	2.0
Cyanidin-3-arabinoside	1.4
Cyanidin-3-glucoside	0.1
Peonidin-3-galactoside	2.8
Peonidin-3-arabinoside	1.1
Peonidin-3-glucoside	0.3

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individual anthocyanins is very consistent and quite indicative of the quality of standard cranberry juice.

In addition to the anthocyanins, epicatechin (procyanidin monomer) was also quantified in this analysis of cranberry juice cocktail and found in an amount of 3.5 mg/L with a 27 percent coefficient of variation.

Flavonols

The flavonols also contribute to the color of the fruit and juice. The flavonols impart a yellowish color in solution and can form co-pigments with the anthocyanins. Figure 4 shows the basic structure of the flavonol aglycone, where hydroxyl substitutions are seen at positions 3' and 5' and glycosidic substitutions at position 3.

The flavonols are also reported to have a number of biological activities including antioxidant, analgesic, radical scavenging, anti-inflammatory, bronchodilator, as well as potential impact on cardiovascular health through improved capillary fragility.

The data presented in Table VI were obtained using the same method and set of samples as for the anthocyanin data presented above. Quantification was achieved using individual external standards of each flavonol and the results reported on an as tested basis. The coefficient of variation for the results ranged from 9 to 26%. Quercitrin, hyperoside and avicularin are all glycosides of the aglycone quercetin. No myricetin glycosides were detected in this analysis.

Phenolic Acids

The phenolic acids are also of growing interest for their potential health benefits. Figure 6 shows the structures for benzoic and cinnamic acids (R=H). Benzoic and cinnamic acids are the two main phenolic acids found in cranberry, along with their derivatives, which are formed by hydroxy and methoxy substitutions at positions 3, 4 and 5.

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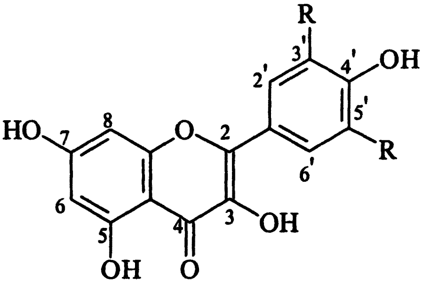

Figure 4. Flavonol Aglycone.

Table VI. Flavonol Content of Cranberry Juice Cocktail (mg/L)

Hyperoside	23.2
Quercetin	13.0
Myricetin	5.3
Quercitrin	5.2
Avicularin	1.8

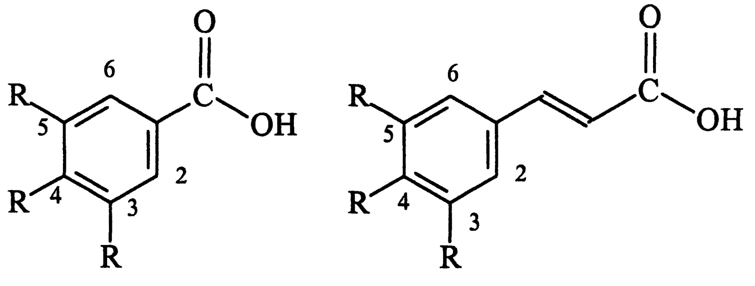

Figure 6. Phenolic Acid Structures.

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The phenolic acids are reported to have a number of biological activities including antioxidant, radical scavenging, platelet aggregation inhibition, plaque formation suppression, anti-hypercholesterolemic, anti-microbial and anti-ulcer.

For the phenolic acid data presented in Table VII, a RP HPLC ES/MS method was developed, using MS/MS mode. Analyte specificity was achieved based on a unique combination of retention time and a parent/daughter ion pair selected for each phenolic acid. The same set of 12 CJC samples described above were chromatographed on a C18 column using a binary gradient of 1% acetic acid and 100% methanol. Quantification was achieved using individual standards calibrated against an internal standard (4-chlorobenzoic acid). The results are again reported on an as tested basis. The coefficient of variation for the results ranged from 20 to 36%. Vanillic acid is a benzoic acid derivative, while caffeic and cholorgenic acid are cinnamic acid derivatives.

Table VII. Phenolic Acid Content of Cranberry Juice Cocktail (mg/L)

Benzoic	43.7
Chlorogenic	11.0
4-Hydroxycinnamic	4.4
3,4-Dihydroxybenzoic	2.3
Vanillic	1.2
Caffeic	1.1

Other Components

In addition to the cranberry constituents just described, there are a number of other components present in cranberries with potential health benefits. A cursory discussion of these components is provided below.

Pectin is a source of fiber, which has been reported to have anticancer and cholesterol lowering activity. Pectin is also a very important ingredient in the processing of cranberries into cranberry sauce, for its gelling property. As with PACs, pectin also presents significant challenges to quantify and qualitatively describe.

Ellagic acid has been reported in cranberry at 120 mg/kg, on a dry weight basis (*32*). Ellagic acid is reported in the literature to have antioxidant, anti-mutagen, anti-carcinogen, and anti-viral activities.

Resveratrol is another compound reported in cranberries, with concentrations similar to that of grape juice at 1.07 and 1.56 nmol/g, respectively (*33*). Resveratrol is associated with anticancer activity (COX1 and COX2 inhibition), is an antioxidant, and is reported as the red wine component responsible for good cardiovascular health, based on its ability to inhibit platelet aggregation and its anti-inflammatory activity.

Lignans are a class of compounds in cranberry seeds that structurally resemble estrogens. The lignan secoisolariciresinol (SECO) has been reported in cranberry at a level of 10.54 mg/kg, on a dry weight basis (*34*). Lignans can function as weak estrogens and estrogen antagonists. In addition they exhibit antioxidant, anti-mitotic, antiviral and anti-tumor activity. Experimental studies in animals have suggested that they are dietary factors that can protect against atherosclerotic vascular disease, by lowering total and LDL cholesterol.

Ursolic acid is found in relatively high concentrations in the cranberry cuticle and that has been used in a salve for skin lesions and burns (*35*). Experimental studies in animals and humans have shown ursolic acid and other triterpenes to exhibit significant biological activities, including hepatoprotective, anti-inflammatory, anti-tumor, anti-hyperlipemic, antiulcer, antimicrobial and anticariogenic.

Tocotrienols and omega-3 fatty acids have also been isolated and identified in cranberry seed oil (*36*). These classes of compounds are known for among other things their antioxidant activity and their impact on carcinogenesis and cardiovascular health.

Compositional Variability

When discussing the chemical composition of cranberries and cranberry products, variability can be expected as for any agricultural commodity. This variability is due to many different factors, such as: variety, maturity, growing location, environmental stresses and processing effects. For example, the mean

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total anthocyanin content (mg/100g) of harvested cranberry fruit significantly varied from 31 to 62 by region, 18 to 66 by variety and 36 to 42 by year, during a six year study of crop quality (unpublished data). The process of depectinization might affect the galacturonic acid content of cranberry juice, among others.

Summary

While cranberries and fruits in general provide many nutritional and health promoting qualities, due to their unique PAC composition cranberries also provide an additional benefit in the form of bacterial anti-adhesion activity. This activity is being seen to have an important impact on urinary tract health, and may impact other health states where bacterial adhesion is part of the disease mechanism.

Continued research on the nutritional and healthful components of cranberries will be challenging. Analytically, lack of standards makes it difficult to quantify some of these components for determining how they vary and how they may be affected by processing. Improved methods are also needed to analyze the complex fractions derived from bioassay directed fractionation. Biologically, there is a need to better understand how these components are absorbed and metabolized for determining the mechanisms involved and so markers can be identified that allow for improved monitoring of clinical study compliance.

Acknowledgements

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Chapter 5

Flavor Compunds of Noni Fruit (*Morinda citrifolia* L.) Juice

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Morinda citrifolia (Rubiaceae), commonly known as noni, is a plant typically found in the Hawaiian and Tahitian islands. It is believed to be one of the most important plants brought to Hawaii by the first Polynesians. The yellow fruits have a dinstinctive "grenade-like" shape and can grow to a size of 12 cm in diameter. It has a foul taste and a soapy smell when ripe. The juice of noni fruit has been shown to prolong the life span of mice implanted with Lewis lung carcinoma. It was proposed that the fruits of noni might suppress the growth of tumors by stimulating the immune system. In recent years, noni juice has been sold in the US market as a nutraceutical supplement. The volatile aroma compounds identified in the fresh and riped noni fruits is described here.

Morinda citrifolia (Rubiaceae), commonly known as noni, is a plant typically found in the Islands of Hawai and Tahiti. It is believed to be one of the most important plants brought to Hawaii by the first Polynesians (*1*). The plant is a small evergreen tree, growing in open coastal regions and in forest areas, up to about 1300 feet above sea level. This plant is identifiable by its straight trunk, large green leaves and its distinctive "grenade-like" yellow fruit. The fruit can grow to a size of 12 cm in diameter and results from coalescence of the inferior ovaries of many closely packed flowers. It has a foul taste and a soapy smell when ripe. The bark, stem, root, leaf, and fruit have been used traditionally as a folk remedy for many diseases including diabetes, hypertension, and cancer (*2,3*). Currently, noni fruit juice is sold as a nutraceutical and dietary supplement.

In earlier studies several nonvolatile compounds including acetyl derivatives of asperuloside, glucose, caproic acid and caprylic acid were identified in fruits (*1*). In recent studies (*4,5*), several new glycosides were isolated and identified from Hawaii noni fruits. Figure 1 shows the structures of these new compounds, **NB-1, NB-2, NB-3, NB-4** and **NB-10**. These glycosides contain hydrolyzable fatty acids, such as octanoic acid and hexanoic acid, and alcohols, such as, 3-methyl-3-buten-1-ol.

There is only one published paper concerning volatile compounds of noni fruit (*6*). A total of 51 detectable volatiles from ripe noni fruit were reported. Twenty acids representing 83% of the total volatiles were identified. It is interesting to note that among them, octanoic (58 %) and hexanoic acids (19 %) dominated the volatile profile. In this study, compare the constituents of noni fruit volatiles collected by SPME (solid phase microextraction) and steam distillation methods were compared.

Materials and Methods

Sample Preparation

Solid-Phase Microextraction (SPME): Noni fruit (340g) were blended and then mixed with 100 mL of water and 150 g of sodium chloride. The temperature of the water bath was 50 °C.

Steam distillation: Noni fruit (340) was blended and mixed with 300ml and 150 g of sodium chloride. The volatiles were dissolved in hexane for GC/AED or GC/MS analysis.

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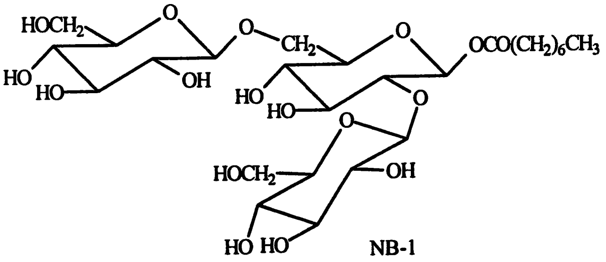
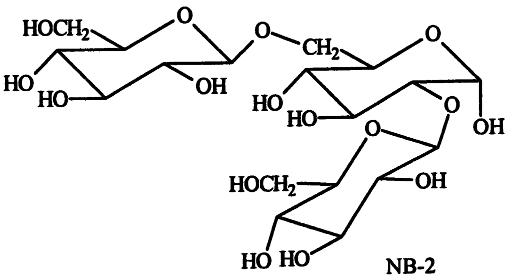


NB-1



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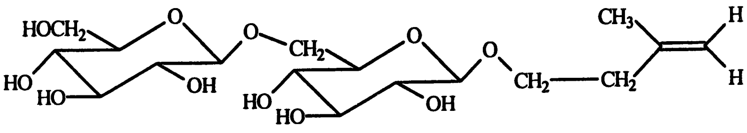

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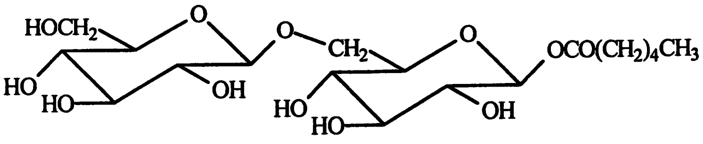

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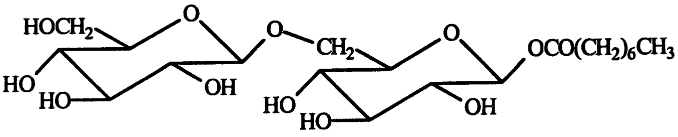

NB-10

Figure 1. Glycosides identified from Hawaiian noni fruits.

SPME Analysis

The SPME holder, SPME fibers (75 μm Carboxen/PDMS) and a 0.75 mm id inlet liner were purchased from Supelco, Inc. (Bellefonte, PA). The sampling time was 30 min and desorption time was 2 min.

GC/MS Analysis. GC/MS analysis was performed on an Agilet 5973 GC/MS equipped with a fused silica capillary column (Supelcowax-10, Supelco, 30 m x 0.25 mm i.d. x 0.25 mm film thickness). The injector temperature was 275°C. The GC oven temperature was programmed as follows: 40°C for 10 min, increased to 240°C at a rate of 4°C/min, and held at 240°C for 10 min. A 0.75 mm id inlet liner in the GC injection port was used for SPME, instead of a larger volume 2 mm id liner.

GC/AED Analysis. An Agilet 6890 GC coupled with a G2350A atomic emission detector was employed for GC/AED analysis.

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Results and Discussion

Figure 2 shows the SPME-GC/AED profile of noni volatiles and Figure 3 shows the GC/AED profile of volatiles isolated from noni by steam distillation. In both Figures, the top panel is the profile obtained from the C channel and the bottom panel is the profile obtained from the S channel. AES (atomic emission spectroscopy) used for this study is a method to identify an atom by measuring the emission of a photon of radiation released by the electrons of the atom after they are excited to a higher energy level (*7*). With the combination of GC-AES, it is possible to detect elements in compounds leaving the column. In the interface, the eluent is atomized and excited by microwave-energized helium plasma that is coupled to a diode-array optical emission spectrometer. Because of the high sensitivity of the detector, we were able to identify several minute quantities of sulfur-containing compounds previously not identified in noni fruit.

Table 1 lists the volatile compounds identified in noni fruit juice. The most abundant compounds were octanoic and hexanoic acids, as well as their corresponding methyl and ethyl esters. In a previous report, we indicated the isolation of a tridisaccharide fatty acid ester, 2,6-di-*O-(β-D-glucopyranosyl)-1-O- hexanoyl-β-D-glucopyranoside* and two disaccharide fatty acid esters, 6-*O-(β-D-glucopyranosyl)-1-O-hexanoyl-β-D-glucopyranoside* and 6-*O-(β-D-glucopyranosyl)-1-O-octanoyl-β-D-glucopyranoside* (Figure 1) from *the* Hawian noni fruit (*4,5*). It is obvious that the sugar esters of fatty acids are the precursors of these acids and esters in noni fruit. They are also the major contributors to the soapy aroma of the ripe noni fruit. Besides hexanoic acid and octanoic acid, a series of medium chain fatty acids such as heptanoic, nonanoic and decanoic

Table I. Volatile compounds identified from ripe fruits of *Morinda citrifolia*

Compounds	*Steam distillation*	*SPME*
Acids		
Formic acid	trace	+
Acetic acid	trace	+
Butanoic acid	+	+
Hexanoic acid	+++	++++
Heptanoic acid	+	+
Octanoic acid	++++	++++
2-Octenoic acid	+	+
Nonanoic acid	+	+
Decanoic acid	+	+
Aldehydes and Ketones		
Acetaldehyde	n.d.	+
2-Methylbutanal	+	+
3-Methylbutanal	+	+
2-Pentanone	+	+
3-Methyl-2-butanone	+	+
2-Hexanone	+	+
Hexanal	+	+
2-Heptanone	+	+
2-Hexenal	+	+
Furfural	+	+
Benzaldehyde	+	+
Alcohols		
Ethanol	+	+
2-Methyl-3-buten-1-ol	+	+
1-Butanol	+	+
3-Methyl-3-buten-1-ol	+++	++
3-Methyl-2-buten-1-ol	+	+
Benzyl alcohol	n.d.	trace
Esters		
Ethyl acetate	+	+
Butyl acetate	+	n.d.
Methyl 2-methylpropanoate	+	+
Methyl butanoate	+	+
Ethyl butanoate	+	+
Butyl butanoate	+	+
4-Pentenyl butanoate	+	+
Methyl 3-methylbutanoate	+	+
3-Methyl-3-buten-1-yl 3-methylbutanoate	+	+

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Table I continued.

Methyl 2-methylbutanoate	+	+
Methyl hexanoate	++	+++
Ethyl hexanoate	++	++
Butyl hexanoate	+	+
4-Pentenyl hexanoate	+	+
3-Methyl-3-buten-1-yl hexanoate	+	+
Hexyl isovalerate	+	+
Methyl heptanoate	+	+
Methyl ocatanoate	++	++++
Ethyl octanoate	+	+
Butyl octanoate	+	+
3-Methyl-3-buten-1-yl octanoate	+	+
Methyl 2-octenoate	+	+
Methyl 3-octenoate	+	+
Methyl 6-octenoate	+	+
Methyl nonanate	+	+
Methyl 5-nonenoate	+	+
Methyl decanoate	++	++
Ethyl decanoate	+	+
Methyl 4-decenoate	+	+
Ethyl 4-decenoate	+	+
Methyl salicylate	+	+
Methyl hexadecanoate	+	+
Terpenes		
Linalool oxide	+	trace
(*Z*)-3,7-Dimethyl-1,3,6-octatriene	+	trace
(+)-4-Carene	+	trace
D-Limonene	+	trace
Ocimenol	+	trace
Terpineol	+	trace
Sulfur Compounds		
Methanethiol	n.d.	+
S-Methyl thioacetate	+	+
Dimethyl disulfide	+	+
Methyl 3-methylthiopropanoate	+	+
Ethyl 3-methylthiopropanoate	+	+
Methionic acid	+	trace

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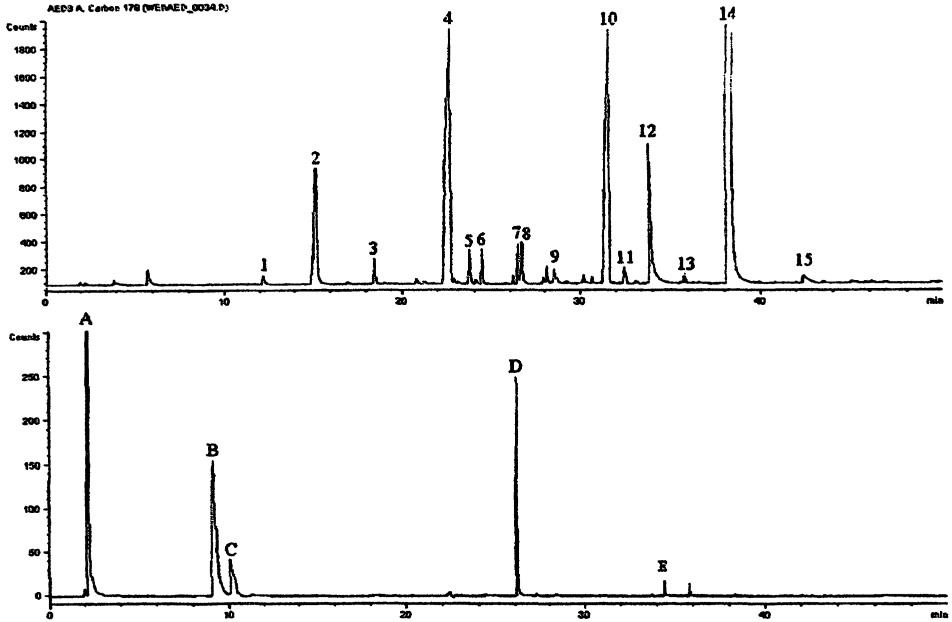

Figure 2. SPME-GC/AED profile of Noni: Top C-channel, bottom S-channel. 1. methyl butanoate; 2. methyl hexanoate; 3. ethyl hexanoate; 4.methyl octanoate; 5. ethyl octanoate; 6. 3-methyl-3-butenyl 3-methyl 3-butenoate; 7. methyl decanoate; 8. ethyl decanoate; 9. 2-methylbutanoic acid; 10. 3-methyl-3-butenyl octanoate ; 11.5-methyl-5-hexenoic acid; 12. hexanoic acid; 13. heptanoic acid; 14. octanoic acid; 15. decanoic acid; A. methanethiol; B. S-methyl thioacetate; C. dimethyl disulfide; D. methyl 3-methylthiopropanoate; E. methionic acid.

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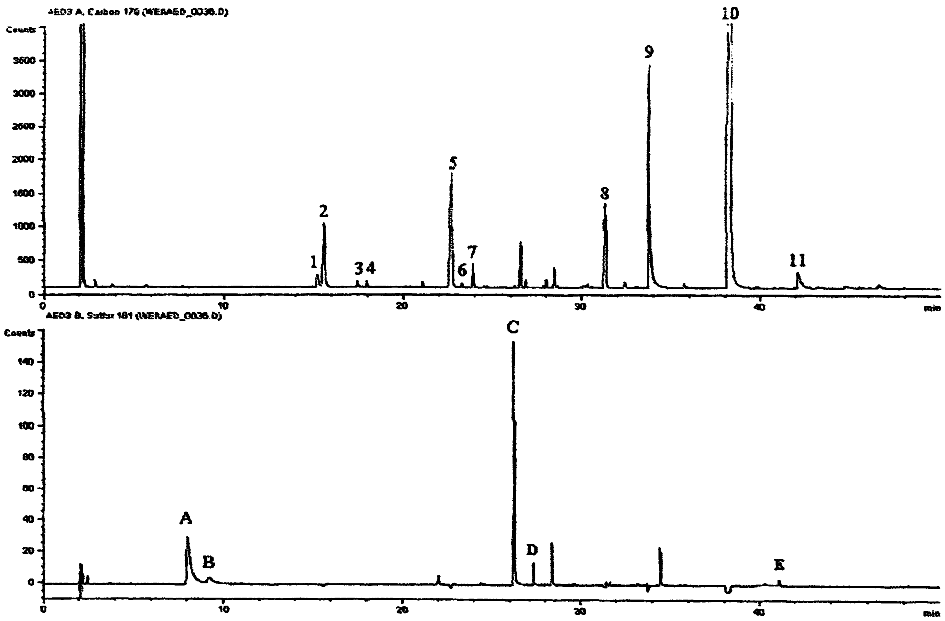

Figure 3. GC/AED profile of volatiles from noni by steam distillation: Top C-channel, bottom S-channel. 1. 2-heptanone; 2. methyl hexanoate; 3. ethyl hexanoate; 4. methyl-3-buten-1-ol; 5.methyl octanoate; 6. butyl hexanoate; 7. ethyl octanoate; 8. 3-methyl-3-butenyl octanoate; 9. hexanoic acid; 10. octanoic acid; 11. decanoic acid; A. S-methyl thioacetate; B. dimethyl disulfide; C. methyl 3-methylthiopropanoate; D. ethyl 3-methylthiopropanoate; E. methionic acid.

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acid were also characterized from both the steam distillate and the headspace of ripe noni fruit.

In addition to sugar fatty acid esters, glycoside of 3-methyl-3-buten-1-ol were also identified in the nonvolatile extract of noni fruits (*4*). In the present study, a relatively large quantity of 3-methyl-3-buten-1-ol was observed in the volatile components of noni fruit juice.

Thirty-two esters were identified in noni juice. It is not surprising that the esters of hexanoic acid and octanoic acid are the major esters identified. Two new esters were tentatively characterized. The mass spectral data of both compounds are very similar. They have a base peak at m/z 68, followed by m/z 57, 127 and 41 fragments. CI-MS data show that these compounds have molecular weights of 182 and 212, respectively. Figure 4 shows the proposed fragmentation pathway for the generation of m/z 41, 57 , 68 and 127 ions. They are also derived from 3-methyl-3-buten-1-ol which exists as one of the major glycosides in noni fruit. The molecular weight of these two compounds have also been reported by Farine *et al.* (*6*) as two unknown compounds representing 0.57 and 1.65% of the total volatiles in ripe noni fruit.

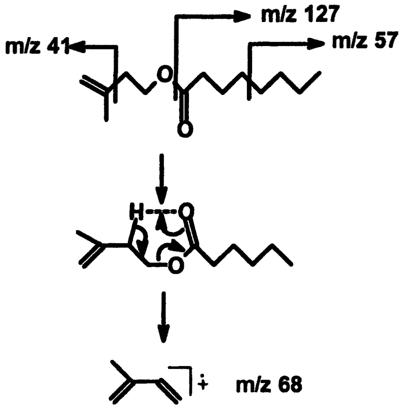

Figure 4. Mass spectral fragmentation pathway for 3-methyl-3-buten-1-yl octanoate.

Because of the sensitive AED detector used, several suifur-containing compounds were identified in noni fruit juice. Interesting thioesters, methyl 3-methylthiopropanoate and ethyl 3-methylthiopropanoate were characterized. As shown in Figure 5, methionine may undergo a transamination to form a keto acid. Subsequent decarboxylatiom leads to the formation of a CoA ester. The release of the CoA moiety results in the generation of methionic acid. Incorporation of methanol and ethanol to the CoA ester may form methyl 3-methylthiopropanoate and ethyl 3-methylthiopropanoate, respectively. The sulfur compounds idenfied may make an important contribution to the flavor of noni fruit juice.

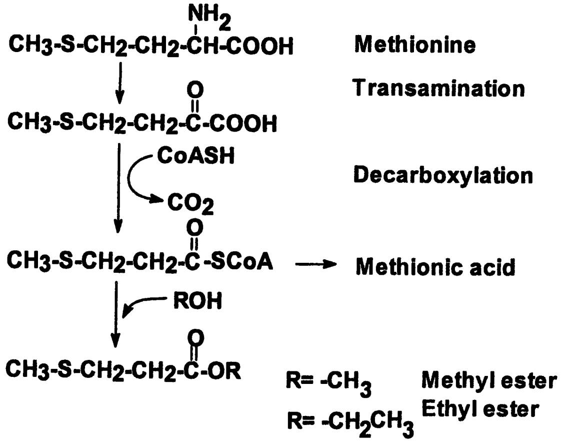
NH2
CH3-S-CH2-CH2-CH-COOH
Methionine
Transamination
O
CH3-S-CH2-CH2-C-COOH
CoASH
Decarboxylation
CO2
O
CH3-S-CH2-CH2-C-SCoA
Methionic acid
ROH
O
CH3-S-CH2-CH2-C-OR
R= -CH3
Methyl ester
R= -CH2CH3
Ethyl ester

Figure 5. Proposed biosynthetic pathway for methyl and ethyl esters of 3-methylthiopropanoate.

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Chapter 6

Cardiovascular Protective Effects of Hawthorn

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Hawthorn has been used as an herbal medicine for treatment of cardiovascular disease in both the east and the west. The hawthorn fruit contains a variety of flavonoids which have been characterized; some of which possess strong antioxidant activity which may account for some of their cardiovascular protective effect. In addition to flavonoids, there are other uncharacterized compounds from the hawthorn fruit which have blood-vessel relaxant and cholesterol lowering activities. The effectiveness of hawthorn in lowering blood cholesterol has been demonstrated in both animal and human studies. The underlying mechanism on the hypocholesterolemic effect of hawthorn appears to be related to LDL receptor regulation as well as modulation of enzyme activities involved in the turnover and transport of cholesterol.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Hawthorn (*Crataegus*) is widely distributed throughout the northern temperate regions of the world with approximately 280 species primarily in East Asia, Europe and North America. The two major species of hawthorn in China are *Crataegus pinnatifida* and *Crataegus cuneata*. They have been used as herbal medicine in China to combat ailments such as scurvy, constipation, blood stasis and indigestion for centuries. In recent years, hawthorn has been used in China as well as Europe for treatment of cardiovascular disease (1,2,3). The beneficial effects of hawthorn appear to be associated with three major biological activities, viz., antioxidant, lipid/cholesterol lowering and blood vessel relaxation. In order to establish the medicinal effect of hawthorn, our research group has carried out extensive studies on the different mechanisms of action of the fruit. In the following sections, we shall present some of our current findings on the different modes of action of hawthorn.

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Antioxidant Activity of Hawthorn

Hawthorn fruits are a rich source of flavonoids (4). Flavonoid consumption has been documented to be negatively associated with coronary heart disease mortality (5). In recent years, it has been generally accepted that oxidation of serum LDL may lead to an increase risk in the development of atherosclerosis (6,7). Hence, one of the beneficial effects of hawthorn may be contributed by its high level of flavonoids as these compounds are powerful antioxidants.

In this study, we characterized the major flavonoids present in the hawthorn fruit using organic solvent fractionation and HPLC techniques. The chemical structures and their contents in the fruit are described in Figure 1 and Table I. Epicatechin and procyanidin B_2 are the most abundant, followed by chlorogenic acid, hyperoside, isoquercitrin, protocatechuic acid, rutin and quercetin. These 8 compounds demonstrated varying degrees of antioxidant activity (Table I). Procyanidin B_2 and hyperoside were most protective to human LDL, followed by isoquercetin and quercitrin. Under the same experimental conditions, the antioxidant activity of epicatechin, rutin, cholorogenic acid and protocatechuic acid was similar but much weaker than that of procyanidin B_2 and hyperoside. Eucomic acid, eucomic acid 4-methyl ester and urosolic acid are all found to be weak antioxidants.

Although a large number of studies have been carried out on the health benefits of hawthorn, no direct study on the absorption and bioavailability of its

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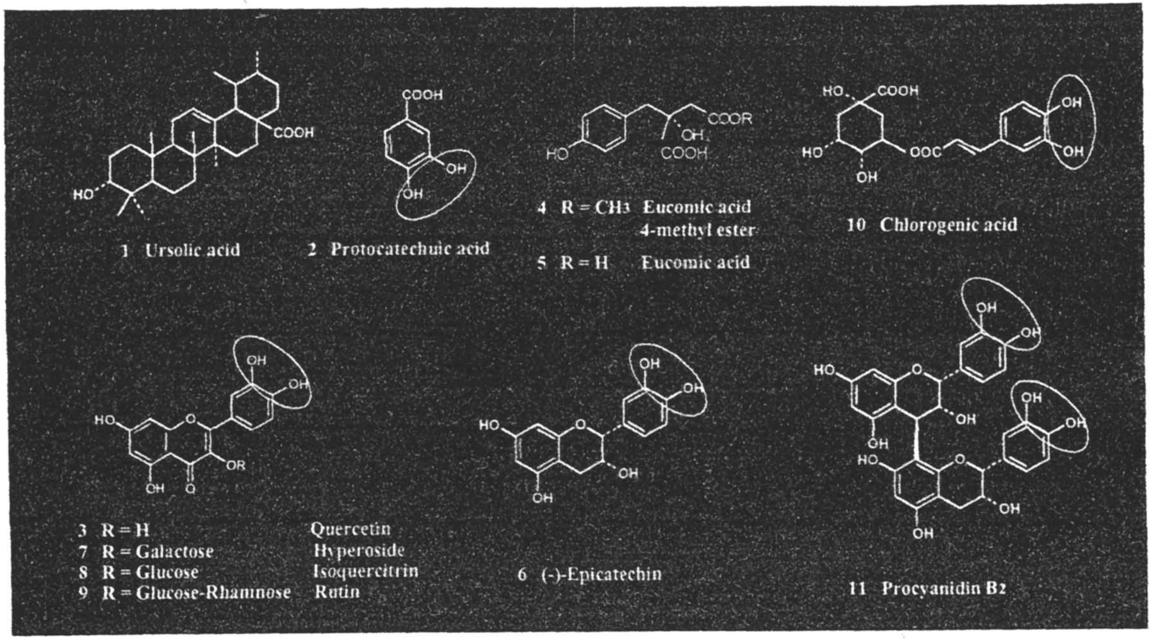
HO
COOH
1 Ursolic acid
COOH
OH
OH
2 Protocatechuic acid
HO
COOR
OH
COOH
4 R = CH3 Eucomic acid 4-methyl ester
5 R = H Eucomic acid
HO
COOH
HO
OOC
OH
OH
OH
10 Chlorogenic acid
HO
O
OH
OH
OR
OH
O
3 R = H Quercetin
7 R = Galactose Hyperoside
8 R = Glucose Isoquercitrin
9 R = Glucose-Rhamnose Rutin
HO
O
OH
OH
OH
OH
6 (-)-Epicatechin
HO
O
OH
OH
OH
HO
O
OH
OH
OH
OH
OH
11 Procyanidin B2

***Figure 1.** Structures of 11 compounds isolated from hawthorn fruit using ethyl acetate extraction and HPLC techniques. The pair of hydroxyl groups circled indicates potential site of anti-oxidant activity.*

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Table I. Flavonoid Content of Hawthorn Fruit and Their Protective Activities on LDL Oxidation

Compound	*IC_{50} Value on LDL Oxidation (μM)*	*Content in Fruit (mg/100g, n=3)*
Procyanidin B_2	0.75 + 0.03	194.02 + 4.28
Hyperoside	0.95 + 0.02	24.56 + 1.06
Isoquercitrin	1.14 + 0.01	16.44 + 0.51
Quercetin	1.17 + 0.02	0.88 + 0.05
Epicatechin	1.38 + 0.01	178.27 + 6.54
Rutin	1.40 + 0.02	2.57 + 0.06
Chlorogenic acid	1.50 + 0.01	64.86 + 2.02
Protocatechuic acid	2.63 + 0.02	3.21 + 0.06
Eucomic acid	> 100	nd
Eucomic acid 4-methyl ester	> 100	nd
Ursolic acid	> 100	nd
Ascorbic acid (positive control)	37.6 + 0.28	

Note: The LDL oxidation protection assay was performed by the TBARS method as described in reference 21. nd, not determined.

active components has been investigated. To correlate the pharmacological action of the hawthorn flavonoids with their potential health benefit, we studied the absorption kinetics and excretion of epicatechin, chlorogenic acid, hyperside and isoquercitrin after oral administration in rats. For comparison, the individual pure compounds as well as a hawthorn phenolics extract (HPE) were studied. Rats were randomly divided into 6 groups. In Groups 1 and 2, the rats received HPE at a single oral dose of 220 mg/kg for determination of epicatechin (Group 1) and chlorogenic acid, hyperoside and isoquercitrin (Group 2) simultaneously. The separate determination of epicatechin from others was needed due to the highly different pharmacokinetics between epicatechin and the three other flavonoids. For rats in Groups 3, 4, 5 and 6, the pure compounds were administered individually at a single oral dose of 34.8 (epicatechin), 4.5 (isoquercitrin), 7.5 (chlorogenic acid) and 6.0 (hyperoside) mg/kg, respectively. These amounts were equivalent to the individual flavonoid content administered as HPE in Groups 1 and 2.

As chlorogenic acid and hyperoside could not be detected in the plasma, urine or feces after oral administration, their pharmacokinetics parameters could not be assessed. The pharmacokinetic parameters of epicatechin and isoquercitrin, after oral administration in an extract mixture or pure form are shown in Table II. No significant difference in kinetic parameters were noticed when epicatechin and isoquercitrin were administered in either a mixture

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Table II. Pharmacokinetic Parameters of Isoquercitrin and Epicatechin in Rats Following Oral Administration

Compound Administered	T_{max} (min)	C_{max} (μg/ml)	$T_{1/2, \lambda 2}$ (min)
Isoquercitrin (n = 9)			
Pure form (4.5 mg/kg)	9.4 ± 1.7	1.31 ± 0.45	10.0 ± 2.0
Mixture	10.6 ± 1.7	0.92 ± 0.34	12.3 ± 2.7
Epicatechin (n = 7)			
Pure form (34.8 mg/kg)	64 ± 11	1.60 ± 0.53	71 ± 13
Mixture	68 ± 14	1.26 ± 0.28	78 ± 14

Note: The number of animals used in the experiments is indicated by the value n. Value presented are mean ± SD.

formulation or a pure form. Isoquercitrin was absorbed and entered the systemic circulation very rapidly with a T_{max} of approximately 10 min. In contrast, epicatechin was absorbed much more slowly reaching a T_{max} at 66 min. The absolute bioavailability of epicatechin and isoquercitrin were 34 and 61%, respectively, as estimated from our data.

Based on the above limited study, it is apparent that different flavonoids from hawthorn may have very different oral absorption and clearance characteristics when administered to either animals or human. More detailed investigations are needed to delineate the pharmacological benefits of these flavonoids as some of them might have limited bioavailability. Structurally, isoquercitrin and hyperoside are very similar except the former one is a glucoside and the other is a galactoside (Figure 1). Yet, isoquercitrin is absorbed into the bloodstream quickly while hyperoside is not. The reason for this observation is not clear at the moment. Out of the four compounds studied, isoquercitrin was observed to be least stable when incubated in the presence of small intestinal and colon contents. In contrast, all compounds were stable in the presence of stomach contents. Thus, the difference in absorption between hyperoside and isoquercitrin is likely due to rapid uptake of the latter in the stomach and unlikely due to degradation of the compounds in the GI tract. Moreover, we have also evaluated whether hyperoside was absorbed in a modified chemical form by measuring the concentration of quercetin in the plasma after enzymic hydrolysis. The result showed that no quercetin was detected. Hence, it is likely that certain flavonoids may be preferentially uptake or degraded in the GI tract while others may not.

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Blood Vessel Relaxant Properties of Hawthorn

Hawthorn extract is well recognized in Europe as an antihypertensive remedy, particularly useful in the treatment of mild forms of cardiac insufficiency and angina pectoris which are usually associated with decreased coronary blood flow (3). Animal studies with the isolated heart have verified that hawthorn extract increased coronary blood flow (8). The cardioprotective effect of hawthorn extract was also reported on the ischemic-reperfused rat heart (9). This protection may be partly associated with the anti-oxidative activity of hawthorn extract, which inhibits formation of free radicals and subsequent damage to the cardiac tissue (10,11).

We have recently demonstrated that hawthorn extract induced concentration-dependent relaxation primarily through endothelium-dependent action in rat isolated mesenteric arteries and aortas. Our results showed that the relaxant effect of hawthorn extract in rat mesenteric arteries was concentration dependently reduced by N^G-nitro-L-arginine methyl ester, a competitive inhibitor of nitric oxide synthase or by methylene blue, an inhibitor of guanylate cyclase. Pretreatment of endothelium-intact artery rings with L-arginine, the nitric oxide precursor, partially reversed the effect of N^G-nitro-L-arginine methyl ester (12). Neither indomethacin nor glibenclamide affected the relaxant response to hawthorn extract, indicating that relaxing prostanoids or activation of ATP-sensitive K^+ channels are not involved. The endothelial nitric oxide-mediated relaxation induced by hawthorn extract was further supported by the stimulatory effect on tissue content of cyclic GMP in endothelium-intact rat aortic rings. Hawthorn extract dose-dependently increased cyclic GMP levels and this effect was completely abolished following pretreatment with N^G-nitro-L-arginine (another nitric oxide synthase inhibitor) or 1H-[1,2,4]oxadiazolo[4,2-α]quinoxalin-1-one (selective guanylate cyclase inhibitor) or in the absence of the functional endothelium (unpublished results). Pretreatment with small doses of hawthorn extract significantly enhanced the relaxant response to acetylcholine, an endothelium/nitric oxide-dependent vasodilator. However, we also found a differential role of endothelial nitric oxide in hawthorn fruit extract-induced relaxation of other three different rat arteries. In cerebral, coronary and carotid arteries, the endothelium does not seem to participate in the extract-induced relaxation since neither N^G-nitro-L-arginine methyl ester nor 1H-[1,2,4]oxadiazolo[4,2-α]quinoxalin-1-one altered the relaxant effect of hawthorn extract. In contrast, endothelial nitric oxide mediates the extract-induced endothelium-dependent relaxation in rat aorta (Chan *et al.*, unpublished observation). It is suggested that some active ingredients in hawthorn extract may have a direct muscle relaxant effect, e.g., possible inhibition of Ca^{2+} influx in vascular smooth muscle cells (12).

The reported anti-oxidant activity of hawthorn fruit (13), the relaxing effect on the carotid, cerebral and coronary arteries suggests a potential preventative effect of hawthorn fruit against cerebral or coronary circulation-related disease such as cerebral vasospasm and stroke. The endothelial nitric oxide-dependent effect indicates that hawthorn fruit extract (12) may possess a broad spectrum of beneficial effects on the cardiovascular function.

Hypocholesterolemic Activity of Hawthorn

Animal Studies

Hawthorn fruit is beneficial to the cardiovascular system, partially due to its effect on serum cholesterol. To investigate the mechanisms by which hawthorn fruit decreases serum cholesterol, two animal models, using the rabbit and

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hamster, were used in the present study. First, the effect of hawthorn fruit supplementation on the accumulation of cholesterol in different organs were quantified. Second, we sought to determine whether supplementation of hawthorn fruit would lead to any changes in major enzyme activities, viz., liver 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) redutase, liver cholesterol-7α-hydroxylase (CH) and intestinal acyl CoA:cholesterol acyltransferase (ACAT), which may affect cholesterol turnover or metabolism. The effect of hawthorn supplementation on fecal excretion of acidic and neutral sterols was also examined.

Rabbits

New Zealand white rabbits (n=24, 3.8-4.4kg) were divided into three groups and housed in an animal room at 25 °C with 12:12-h light-dark cycles. The first group (n=4) was fed a reference diet (Glen Forrest Stockfeeds, Western Australia, Australia) that contained no added cholesterol (NC). The second group (n=10) was fed a high-cholesterol diet (HC) that was prepared by adding1.0g cholesterol per 100 g of the NC diet. The third group (n=10) was fed a HC diet supplemented with 2.0g/100g hawthorn fruit powder (HC-H). All the rabbits were killed after overnight food deprivation under carbon dioxide anesthesia and the blood was collected. The total fecal output of each rabbit was combined for whole week 12. The organs including liver, heart, and kidney were removed, washed with saline, and stored at - 80°C. The thoracic aorta from the aortic bulb to the branching of the celiac artery was then removed and saved for measurement of cholesterol.

Serum total cholesterol (TC) and triacylglycerol (TG) levels in the HC-H was 23.4% and 22.2% lower, respectively, than those in the HC rabbits ($p<0.05$, Table III). In contrast, serum HDL-cholesterol (HDL-C) level in HC-H rabbits was significantly higher than that in HC group ($p<0.05$). When the hawthorn fruit was supplemented in diet, the accumulation of cholesterol in HC-H rabbits was significantly reduced to 13.9±8.0 µmol/g aorta compared with that of the HC group (28.3±14.3 µmol/g, $p<0.05$). No significant differences in the aortic TG level were observed among the three groups although the TG in the HC and HC-H was slightly higher than the NC (Table III). The cholesterol accumulation in the liver reached 95.0± 24.2 µmol cholesterol /g liver in the HC group but when hawthorn fruit was supplemented in the diet, it was reduced to 58.0± 14.4 µmol /g ($p<0.05$). Similarly, the hawthorn group had lower levels of cholesterol in both heart and kidney than the HC rabbits ($p<0.05$). The analysis of feces showed that the HC-H group had higher excretion of both neutral and acidic sterols compared with the HC rabbits ($p<0.05$).

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Table III. Effect of Hawthorn on Serum, Organ and Fecal Cholesterol Level of Rabbits Fed a High Cholesterol Diet

	NC	*HC*	*HC-H*
Serum			
Total cholesterol (mmol/L)	0.5 ± 0.2^{c}	24.7 ± 2.8^{a}	18.9 ± 4.7^{b}
HDL-cholesterol (mmol/L)	0.3 ± 0.1^{a}	0.2 ± 0.1^{b}	0.3 ± 0.1^{a}
Triacylglycerols (mmol/L)	0.6 ± 0.1^{c}	2.2 ± 0.5^{a}	1.7 ± 0.3^{b}
Aorta			
Total cholesterol (μmol/g)	1.5 ± 0.7^{c}	28.3 ± 14.3^{a}	13.9 ± 8.1^{b}
Triacylglycerols (μmol/g)	31.8 ± 2.8^{b}	52.0 ± 24.2^{a}	49.6 ± 24.8^{a}
Liver total cholesterol (μmol/g)	3.0 ± 0.4^{c}	95.0 ± 24.2^{a}	58.0 ± 14.4^{b}
Heart total cholesterol (μmol/g)	2.8 ± 0.3^{c}	7.4 ± 2.4^{a}	5.5 ± 0.9^{b}
Kidney total cholesterol (μmol/g)	6.9 ± 0.6^{c}	17.7 ± 1.8^{a}	14.2 ± 2.6^{b}
Fecal total neutral sterols (mg/g)	51.1 ± 10.1^{c}	134.1 ± 19.6^{b}	264.4 ± 36.1^{a}
Fecal total acidic sterols (mg/g)	13.2 ± 2.1^{c}	18.1 ± 2.4^{b}	35.5 ± 4.8^{a}

Values are means±SD, ^{a,b,c}Means at a row with different letters differ significantly, $P<0.05$. NC: a reference diet; HC: a 1% cholesterol diet; HC-C: a 1% cholesterol diet supplemented with 2.0% dry hawthorn fruit powder. See text for experimental details.

Hamsters

Male Syrian golden hamsters (n=30, 95-110 g) were randomly divided into two groups fed either a control diet or a hawthorn diet. The control diet was prepared by mixing the following ingredients: casein, 200 g; lard, 200 g; cornstarch, 418 g; sucrose, 100 g; AIN-76 mineral mix, 40 g; AIN-76A vitamin mix, 20 g; DL-methionine, 1 g; and cholesterol, 1 g. The hawthorn diet was similar to the control except that 0.5% hawthorn fruit ethanolic extract (equivalent to 2% dry hawthorn fruit powder) was added. Both the control and the hawthorn diets (1 kg) were then mixed with 300 mL of gelatin solution (20 g/L). Once the gelatin had set, the food was cut into ~20 g cubed portions and stored in a freezer at –20 °C. Frozen diets were given to hamsters daily, and uneaten portion was discarded. The total fecal output of each hamster was combined during entire week 4. All hamsters were killed after food deprivation for 14 h. The blood was collected via the abdominal aorta. The liver was removed, washed with saline, and stored at –80 °C for assays of 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase and cholesterol-7α-hydroxylase (CH) activity. The intestine was also saved; the first 10 cm from the stomach was discarded, and the next 30 cm taken for the intestinal acyl CoA:cholesterol acyltransferase (ACAT) determination.

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Significant reductions in the serum TC by 10% and TG by 13% were observed in the hawthorn group compared with the control hamsters ($p<0.05$, Table IV). However, supplementation of hawthorn fruit ethanolic extract had no effect on the serum HDL-C level. The hawthorn-supplemented group had higher fecal excretions of both neutral and acidic sterols *per se* during week 4 compared with the control group ($p<0.05$). The activity of liver HMG-CoA reductase in the hawthorn-supplemented group was not different from that of the control group. However, the activity of liver CH in the hawthorn-supplemented group was significantly increased when compared with that for the control group ($p<0.05$). The activity of intestinal ACAT in the hawthorn-supplemented group was significantly decreased compared with that of the control group ($p<0.05$).

The present results in rabbits and hamsters confirmed that hawthorn fruit possessed a hypolipidemic and antiatherosclerotic activity. Part of this study has been published elsewhere (14,15) The observation is in agreement with that of Chen *et al* (16), who demonstrated that TC and TG levels were decreased by 15 and 10%, respectively, but HDL-C remained unchanged in 30 hyperlipidemic subjects after consuming hawthorn fruit drinks for one month. In the same report, hawthorn drinks in replacement of tap water was found to reduce both serum TC and TG in rats.

The mechanisms by which dietary hawthorn fruit decreases serum cholesterol are probably attributed to its effect on several metabolic points of cholesterol metabolism including decreased synthesis, activation of LDL receptors, inhibition on absorption of dietary cholesterol, and conversion of cholesterol to bile acids. The decrease in cholesterol biosynthesis would lead to a lower blood cholesterol level. Rajendran *et al.* (17) found that part of the hypocholesterolemic mechanisms of hawthorn fruit was associated with its inhibition on cholesterol biosynthesis in rats. The present study found no effect of consumption of hawthorn fruit ethanolic extract on the HMG-CoA reductase activity in hamsters, suggesting that inhibition of cholesterol synthesis is not mediated by down-regulation of HMG-CoA reductase.

The inhibition of cholesterol absorption in intestine could also reduce serum cholesterol. To study further the effect of hawthorn fruit supplementation on the absorption of cholesterol, the intestinal ACAT activity was measured. This is because intestinal ACAT may play a key role in the absorption of cholesterol by esterification of cholesterol prior to absorption (18). The present result demonstrated supplementation of hawthorn fruit ethanolic extract was associated with a lower intestinal ACAT activity. Greater excretion of bile acids could also lead to a lower level of serum cholesterol. The present results also demonstrated that fecal excretion of both primary (cholic and chenodeoxycholic) and secondary (lithocholic and deoxycholic) bile acids were greater in the hawthorn group compared with that in the control group. The liver CH removes cholesterol and produces 7α-hydroxycholesterol, which is the first step on the

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Table IV. Effect of Hawthorn on Serum Cholesterol, Fecal Sterol and Various Enzyme Activities in Hamsters Fed with a High Cholesterol Diet

	Control (n=15)	*Hawthorn (n=15)*
Serum total cholesterol (mmol/L)	4.6±0.5	4.1±0.5*
Serum HDL-cholesterol (mmol/L)	2.3±0.3	2.4±0.3
Serum triacylglycerol (mmol/L)	3.3±0.7	2.9±0.4*
Fecal total neutral sterols (mg/g)	8.6±1.4	11.8±2.0*
Fecal total acidic sterols (mg/g)	3.3±0.9	4.8±1.0*
HMG-CoA Reductase (pm/min.mg protein)	6.6±2.50	6.4±2.5
CH (pm/min.mg protein)	53.0±29.2	148.9±57.2*
ACAT (nm/min.mg protein)	1.0±0.3	0.8±0.2*

Values are means±SD, *Means at a row differ significantly, $P<0.05$. See text for experimental details.

metabolic pathway from cholesterol to bile acids (19). Supplementation of hawthorn fruit significantly increased the liver CH activity compared with the control group, indicating that the increased excretion of bile acids is partially mediated by up-regulation of this enzyme.

Up-regulation of LDL–receptors is probably an alternative mechanism responsible for the hypocholesterolemic activity of hawthorn fruits. In the study by Rajendran *et al.* (17), supplementation of 0.5mL alcholic extract per 100 g body weight per day for six weeks was associated with a 25% increase in hepatic LDL-receptor activity, resulting in greater influx of plasma cholesterol into the liver. The observation is also supported by the study of Ho *et al.* (20), who investigated the effect of hawthorn fruit extract on HepG2 cells and demonstrated the hawthorn fruit extract could prevent the down-regulation of LDL-receptors by LDL.

The reduction in serum TC by dietary hawthorn fruit is a complex process, which may involve multifaceted interactions of cholesterol metabolism. In addition to the effect on LDL receptors, the hypocholesterolemic properties of hawthorn fruit are possibly mediated simultaneously by down-regulation of intestinal ACAT and up-regulation of hepatic CH, leading a greater excretion of both neutral and acidic sterols.

Human Study

In order to evaluate the clinical efficacy of hawthorn in lowering blood cholesterol, a randomized, double-blinded, placebo-controlled, cross-over study was carried out in a group of 73 mildly hypercholesterolemic patients. Subjects were randomized and assigned to the hawthorn drink or placebo group in a cross-over design. Participants were asked to take a 250 mL hawthorn or placebo drink three times a day for 4 weeks. At the end of this period, a wash-out period of 4 weeks was implemented before the cross-over. Patients' blood samples were taken for examination at baseline, week 4, 9 and 12. In addition to TC, LDL-C, HDL-C and TG, other blood chemistry to assess toxicity were also performed.

The hawthorn drink used in the present study was prepared as follows. Fresh fruits were sort and cleaned and then crushed mechanically in a grinder. Water was added to extract the water-soluble content by a multi-stage counter-current process at 40 to 50 °C over a period of 3 h. The pH of the extract was then adjusted to around 3.5 and the extract was clarified by digestion with pectinase. After digestion, the solution was pressure filtered to remove insoluble materials and sucrose was added to a final total content of approximately 18% (including sugar from fruit). The juice thus prepared was then filtered and heat sterilized (93 °C, 15 s), filled in cans and cooled down. Using this method, 480 kg of water-soluble material was extracted from 7000 kg of fresh hawthorn fruit. The nutritional contents of the drink were, 19% (w/w) carbohydrate, 0.2% (w/w) protein, total calories, 78. Heavy metal and bacterial contents were within international acceptable limits. The placebo drink was formulated with sugar and artificial flavor and coloring resembling the taste and color of the hawthorn drink.

For this study, we have screened a total of 350 subjects and 73 were within the acceptable criteria for inclusion (age, 35 to 69 years; blood cholesterol, 220 to 400 mg/dL; blood triglyceride, $\leq$ 40 mg/dL). The average age of the subjects was 56.6 ± 7.3 years and 35 of them were males and 38 were females. Out of the 73 patients recruited, 7 dropped out of the study for various reasons either or not related to the intake of the trial materials. The final number of subjects finishing the study was 66; 33 in Group A and 33 in Group C. Group A took the hawthorn drink followed by the placebo while Group C took the placebo and then the hawthorn drink.

As indicated in Table V and VI, after the intake of the hawthorn drink for a period of 4 weeks, the serum TC and LDL-C of the subjects were reduced on the average by 7 and 13%, respectively. The level of reduction in both cases was statistically significant ($p < 0.05$). In the placebo treatment, although there was a reduction in 2% of TC and in 5% of LDL-C, these changes were not statistically significant ($p > 0.05$). No significant changes in TG and HDL-C were observed after treatment with hawthorn or placebo. The blood chemistry of all of the subjects was normal before and after the trial; indicating that there was no toxicity from the hawthorn juice.

Compared with the animal study, the reduction of blood cholesterol level in humans after treatment with hawthorn is less prominent. As the design of the two studies were not the same, it is not reasonable to compare the results directly. Nonetheless, both of them indicated the efficacy of hawthorn in lowering blood

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TableV. Serum Total Cholesterol Level (mg/dl) After Intake of Hawthorn Juice or Placebo for 4 Weeks

Group	*Baseline*	*Week 4*	*Difference*	*Significance*
Group A: hawthorn	268 ± 57	247 ± 55	-21 ± 33	$p < 0.05$
Group C: placebo	254 ± 40	252 ± 47	-2 ± 35	ns
	Week 9	*Week 12*	*Difference*	*Significance*
Group A: placebo	235 ± 39	227 ± 41	-8 ± 29	ns
Group C: hawthorn	239 ± 38	223 ± 33	-16 ± 29	$p < 0.05$

cholesterol. As neither triglyceride nor HDL-C changed after treatment with hawthorn, this may reflect the specific mechanisms, as discussed above, by which the active ingredients act on the enzyme systems involved in cholesterol absorption and metabolism. The amount of hawthorn juice taken per day in the present study appeared to be the maximum level which our subjects could tolerate. One complaint most frequently received was stomach discomfort after intake of the juice. To avoid such problems when using hawthorn juice as a health supplement, it is advisable that acidity should be reduced.

Table VI. Serum LDL-cholesterol (mg/dl) After Intake of Hawthorn Juice or Placebo for 4 weeks

Group	*Baseline*	*Week 4*	*Difference*	*Significance*
Group A: hawthorn	186 ± 59	163 ± 54	-23 ± 32	$P < 0.05$
Group C: placebo	168 ± 38	160 ± 38	-8 ± 34	ns
	Week 9	*Week 12*	*Difference*	*Significance*
Group A: placebo	161 ± 39	153 ± 42	-8 ± 24	ns
Group C: hawthorn	159 ± 31	137 ± 24	-22 ± 26	$P < 0.05$

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Chapter 7

Anticancer Activity and Flavonoid Content of Various Citrus Juices

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It is commonly accepted that cancer formation can be prevented by the consumption of certain foods; thus flavonoids in *Citrus* fruits and juices are among the most prominent anti-cancer agents. From a viewpoint of health-promotion by dietary habits, cancer preventative activity in the *Citrus* juices is more important than that found in their inedible parts. However, few studies have focused on the biological activities in *Citrus* juices, possibly because the measurable activities in juices tend to be hindered by the more abundant substances, such as sugars.In order to eliminate the masking, we prepared the readily extractable fractions of *Citrus* juices by adsorbing on porous polymer resin, and successive elution from the resin with ethanol and acetone. Among 34 *Citrus* juices examined, King (*C. nobilis*) exhibited potent differentiation-inducing activity toward HL-60 leukemia cells, and the active principles were identified as four polymethoxyflavones.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Flavonoids are important secondary plant metabolites, and are mainly present in plant tissues in relatively high concentrations as sugar conjugates. Epidemiological studies have indicated that flavonoid consumption is associated with a reduced risk of cancer. *Citrus* is a rich source of flavonoids. Determination of *Citrus* fruits with high concentrations of individual flavonoids is desirable in order to study their biological properties. In a recent literature review on *Citrus* flavonoids, a broad spectrum of biological activity including anticarcinogenic and antitumor activities was discussed (*1,2*).

From a viewpoint of health-promotion by dietary habits, cancer preventative activity in the *Citrus* juices is more important than that found in their inedible parts. However, a few studies have focused on the biologically active substances in *Citrus* juices, possibly because the measurable activity of juice tended to be hindered by the more abundant substances, which had no activity *in vitro*, such as sugars.

This study was intended to survey the manifestation of anticancer activity and the content of major flavonoids in the representative and/or economically important *Citrus* species according to Tanaka's classification. We precisely evaluated the influence of cultivar on flavonoid composition by principal component analysis.

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Experimental

A readily extractable flavonoid fraction was prepared from various *Citrus* juices, involving adsorption on Diaion HP-20 (Mitsubishi Chemicals, Tokyo, Japan), a porous polymer resin, and successive elution from the resin with ethanol and acetone. This method led to substantially decrease in sugar content in that fraction.

Various *Citrus* juices were subjected to screening for anticancer effect, which was assayed by the differentiating activity against human promyelocytic leukemia cells (HL-60), and to high-performance liquid chromatography (HPLC) analysis of 24 flavonoids. The influence of cultivar flavonoid composition was examined using principal component analysis.

Results and Discussion

Screening for HL-60 differentiating activity of *Citrus* juices

The readily extractable fraction with reduced sugar content was prepared from *Citrus* juice. The feasibility of the physiological screening on the readily extractable fraction in order to identify *Citrus* species and cultivars rich in anti-cancer substances was therefore demonstrated.

The readily extractable fractions from 34 *Citrus* juices were examined for their HL-60 differentiating activity by monitoring nitro blue tetrazolium (NBT) reducing cell. Total activity was obtained from a division of the amount of fraction yield by the minimum effective dose, i. e. 200 μg/ml. *C. nobilis* (common name; King, total activity; 5860), *C. bergamia* (common name; Bergamot, total activity; 5120), and *C. limettioides* (common name; Sweet lime, total activity; 4545) exhibited strong activity, whereas *C. depressa* (common name; Shiikuwasha, total activity; 2293) and *C. erythrosa* (common name; Kobeni mikan, total activity; 1157) showed moderate activity (*3*).

The HL-60 cells (*4*), established from an acute myeloid leukemia patient, are blocked at a certain step of the cellular maturation process and displayed a high proliferation ability. Terminal differentiation of HL-60 can be monitored by changes of morphological, biochemical, and immunological properties. Certain compounds, known to be efficacious cancer preventative agents, including interferon, retinoic acid, 1α, 25-dihydroxyvitamin D_3, are potent inducers of HL-60 cell differentiation, and appear to be clinically effective against myeloproliferative disorders and human colon, mammary and lung xenografts and melanoma (*5*).

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Isolation of active compounds from King juice

The readily extractable fraction prepared from 200 mL of King juice was subjected to purification of active principles under a guide of HL-60 differentiating activity. The fraction was partitioned between diethyl ether (Et_2O) and water, and the bioactive Et_2O fraction was chromatographed on a silica gel column eluted with $CHCl_3$. Active fraction was finally purified by HPLC to give four active compounds. These compounds were nobiletin (isolation yield; 12.7 mg), heptamethoxyflavone (30.4 mg), natsudaidain (12.0 mg), and tangeretin (11.8 mg). Nobiletin was identified by direct comparison of nuclear magnetic resonance (NMR) and mass spectral data with authentic compounds; heptamethoxyflavone, natsudaidain, and tangeretin were identified by comparison of ^1H- and ^{13}C-NMR spectral data with those reported in the literature (*2,6*). These four polymethoxyflavones as active principles have already been isolated and identified from the fruit peels of *C. nobilis* as HL-60 differentiation inducer (*7*).

King juice was indicated to be rich in polymethoxyflavones. Test medium of the readily extractable fraction of King contained 1.8 μM of polymethoxyflavones (nobiletin; 0.41 μM, heptamethoxyflavone; 0.76 μM, natsudaidain; 0.33 μM, tangeretin 0.33 μM). Assuming that these polymethoxyflavones had same potency of inducing activity of NBT reduction, the total concentration of polymethoxyflavones was very close to the minimum effective concentration examined for these flavonoids (2.5 μM). We, therefore, considered that these polymethoxyflavones mainly contributed the differentiation inducing activity of the readily extractable fraction of King juice.

The 27 *Citrus* flavonoids listed in Figure 1 were examined for their HL-60 differentiation-inducing activity. The rank order of potency of the flavonoid was judged by the percentage of of HL-60 cell which reduced NBT at a concentration of 10 μM. The decreasing sequence of the differentiation-inducing activity is given as follows; natsudaidain > luteolin > tangeretin > quercetin > apigenin > heptamethoxyflavone > nobiletin > acacetin > eriodictyol > taxifolin. The percentages of NBT reducing cells were 62.5 ± 4.0, 58.5 ± 4.0, 53.0 ± 4.1, 51.8 ± 1.9, 44.8 ± 2.9, 39.3 ± 2.7, 37.3 ± 2.9, 36.8 ± 1.1, 36.3 ± 1.9, and 34.0 ± 1.9, respectively (*8*). HL-60 cells appeared to be differentiated into mature monocyte/macrophages *in-vitro* by tangeretin, quercetin, apigenin, heptamethoxyflavone, nobiletin, acacetin, eriodictyol, and taxifolin, since HL-60 cells treated with these compound showed NBT reducing, non-specific esterase, and phagocytic activities in a dose-dependent manner. Monocyte/macrophage cells can be distinguished from granulocyte by the substrate specificity of their cytosolic esterases; cellular esterases of monocyte/macrophage cell hydrolyze α-naphthyl acetate, whereas granulocyte esterases cleave naphthyl AS-D chloroacetate. Following treatment with tangeretin, quercetin, apigenin, heptamethoxyflavone, and nobiletin at a concentration of 40 μM, more than 60% of HL-60 cells were induced differentiation. Natsudaidain and luteolin were more potent than the other flavonoids, inducing more than 50% of HL-60 cells to be differentiated at a concentration of 10 μM, but slight elevations of specific esterase activity were observed. Natsudaidain and luteolin strictly inhibited proliferation of not only HL-60 but also human lung carcinoma (A549), melanin pigment-producing mouse melanoma (B16 melanoma 4A5), T-cell leukemia (CCRF-HSB-2), and gastric cancer cell, and lymph-node metastasized (TGBC11TKB) cell lines at 40 μM (*9*).

Quantitative analysis of flavonoids in various Citrus fruits

Isolation of polymethoxyflavones as biologically active principles suggested the correlation between the flavonoid content and the differentiating activity in *Citrus* juice. To study this, the polymethoxyflavone contents along with other *Citrus* flavonoids were compared within 66 *Citrus* fruits by HPLC analysis. Hesperidin [548.2 ± 523.7 μg/100 mg dry weight (mean ± standard deviation, n = 66), detected in 64 samples out of 66 samples] was the most abundant flavonoid and widely distributed in the citrus samples studied, followed by

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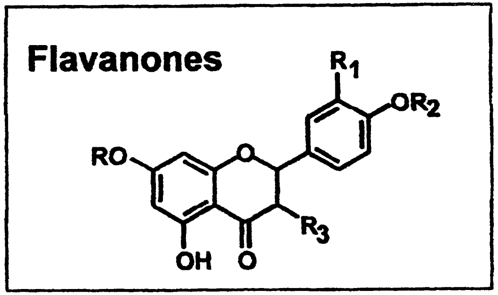
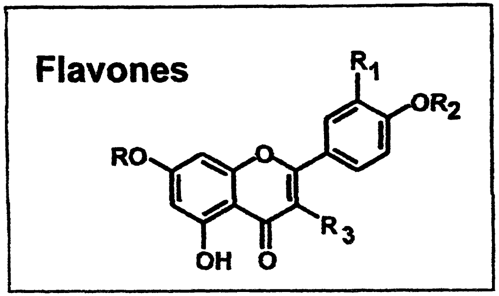


Eriocitrin: R=rutinose, R_1=OH, R_2=R_3=H
Neoeriocitrin: R=neohesperidose, R_1=OH, R_2=R_3=H
Narirutin:R=rutinose, R_1=R_2=R_3=H
Naringin: R=neohesperidose, R_1=R_2=R_3=H
Hesperidin:R=rutinose, R_1=OH, R_2=Me, R_3=H
Neohesperidin: R=neohesperidose, R_1=OH, R_2=Me, R_3=H
Naringenin: R=R_1=R_2=R_3=H
Neoponcirin: R=rutinose, R_1=H, R_2=Me, R_3=H
Poncirin: R=neohesperidose, R_1=H, R_2=Me, R_3=H



Rutin: R=H, R_1=OH, R_2=H, R_3=O-rutinose
Isorhoifolin: R=rutinose, R_1=R_2=R_3=H
Rhoifolin:R=neohesperidose, R_1=R_2=R_3=H
Diosmin: R=rutinose, R_1=OH, R_2=Me, R_3=H
Neodiosmin:R=neohesperidose, R_1=OH, R_2=Me, R_3=H
Quercetin: R=H, R_1=OH, R_2=H, R_3=OH
Luteolin: R=H, R_1=OH, R_2=R_3=H
Kaempferol: R=R_1=R_2=H, R_3=OH
Apigenin: R=R_1=R_2=R_3=H

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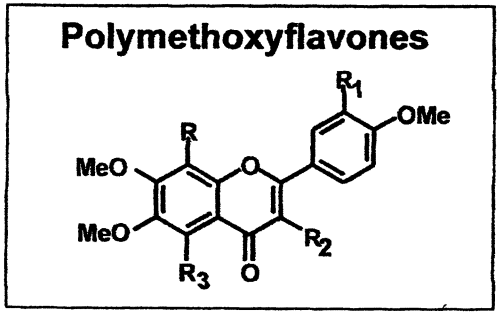

Sinensetin: $R=H$, $R_1=OMe$, $R_2=H$, $R_3=OMe$
Nobiletin: $R=R_1=OMe$, $R_2=H$, $R_3=OMe$
Heptamethoxyflavone:$R=R_1=R_2=R_3=OMe$
Natsudaidain: $R=R_1=OMe$, $R_2=OH$, $R_3=OMe$
Tangeretin: $R=OMe$, $R_1=R_2=H$, $R_3=OMe$

Figure 1. Structures of Citrus *flavonoids studied.*

narirutin and naringin. Hesperidin was not found in Trifoliate orange (*Poncirus trifoliate*), and only small amount was detected in group IV and several species belonging to group V, namely Natsudaidai (*C. natsudaidai*), Kawano Natsudaidai, which is a variation of Natsudaidai, and Sour orange (*C. aurantium*). In contrast, these species contained higher amounts of naringin than those of the high-hesperidin containing species.

Hesperidin and naringin seem to be mutually exclusive, thus they can not exist together at high concentrations. Relationship of this kind has been already reported in several kinds of *Citrus* fruits (*11,12*). Figure 2 also shows the exclusive relationship between hesperidin and naringin contents. The high-naringin containing species are largely group VII species. Exception is the group VI species. These species contain perceptible amounts of both hesperidin and naringin, and seems to form an independent group on the basis of pattern of hesperidin and naringin contents. Luteolin and kaempferol were mainly found in *Fortunella* species.

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Multivariate analysis

The *F* ratio values for each variable by one-way ANOVA are >1 in 21 flavonoids out of 24 flavonoids studied, indicating that these flavonoids show difference among the citrus cultivars examined.

In this study, F-ratio values of naringenin, apigenin, and sinensetin were <1, and therefore these flavonoids were not considered in the next statistical analysis.The data for the 21 flavonoids were used to the principal component analysis, which can reduce the dimensionality of a set of data and thus plotted in conventional two dimensional graphics. The eigenvalues obtained from the correlation matrix were 4.41, 3.06, 2.36, 1.98, 1.51, 1.35, 1.13, 1.07. Choosing only eigenvalues >1 led us to reduction of 21 flavonoids to 8 principal components (PC), according to 80.4% of the total variability. The percentages of variance for the four principal components were 20.99% for the first, 14.55% for the second, 11.25% for the third, and 9.44% for the last one.

The score for the first two principal components was plotted as scatter diagram in Figure 3A. This graphic shows the peculiarities of Trifoliate orange (*Poncirus trifoliate*), Fortunella species, Bergamot, and sour orange. The rest of Citrus species were complicated. After a zoom of the thicket, the distribution of Citrus species belonging to different classes could then be observed (Figure 3B). This graphic shows that five groups, according to Tanaka's classification, could be separated apart from some overlap of groups III, V, and VII. Exceptions were Natsudaidai, Kawano Natsudaidai, and Sudachi (*C. sudachi*). The hybridity of Natsudaidai according to Swingle's classification (*13*) could explain their peculiarities in group V. Numerical taxonomic study also revealed that morphology of Natsudaidai was different from sweet oranges according to Swingle's classification (*14*).

Table I. Contents of Important Flavonoids in the Representative Citrus Juice

Tanaka's Group No.	Conventional name	NGN	HSP	NBL	HPT	NTD	TNG
II	Tahiti lime	0	572	0	2.5	1.1	1.4
II	Bergamot	598	42	0.1	0.2	0.3	0.2
III	Sweet lemon	0	1100	0.1	0	0.2	0
III	Eureka lemon	0	358	0.1	0.1	0.1	0
IV	Hirado	397	0	0.1	0	0	0.7
IV	Marsh grapefruit	1460	0	0.2	0.2	0	0.2
IV	Red blush	1140	0	0.4	0.2	0.1	0.1
IV	Kinukawa	85	0	0.1	0.2	0	0.2
IV	Hassaku	138	33	0.2	0.5	0	0.3
V	Natsudaidai	636	0	0.4	0.5	0.5	1.0
V	Sanbokan	0	190	0.9	1.6	0.5	1.1
V	Sour orange	377	10	0.7	0.5	0.5	0.5
V	Valencia	0	700	1.3	0.4	0.2	0.3
V	Morita navel	0	1080	2.2	0.5	0	0.3
V	Oto Mikan	0	583	0.4	0.2	0	0.3
V	Iyo	0	406	2.0	0.8	0	1.1
V	Hyuganatsu	0	165	1.5	0.9	0	1.3
VI	Yuzu	162	95	0.1	0	0.1	0.1
VII	King	0	1170	1.4	4.0	2.5	2.0
VII	Kunenbo	0	588	1.3	2.0	0	0.9
VII	Sugiyama Unshiu	0	664	0.9	1.2	0	0.5
VII	Okitsu Wase	0	456	0.9	0.9	0	0.4
VII	Yatsushiro	0	549	0.8	1.1	0	0.8
VII	Kabuchi	0	379	5.2	1.3	0	7.4
VII	Ota Ponkan	0	676	5.3	0	0	5.2
VII	Dancy tangerin	0	1510	4.5	0.1	0	1.5
VII	Clementine	0	852	0.8	0.8	0	2.4
VII	Jimikan	0	1330	5.5	0	0	3.2
VII	Shikaikan	0	1200	3.5	0.3	0	1.4
VII	Kobeni Mikan	0	869	5.2	0.1	0	3.5
VII	Hirakishu	0	621	6.7	0	0	3.9
VII	Shiikuwasha	0	992	21	0	0	8.8
VII	Koji	0	180	2.7	0	3.1	2.4
VIII	Shikikitsu	0	50	2.0	0.1	0	1.0

Abbreviations. NGIN; naringin, HSP; hesperidin, NBL; nobiletin, HPT; 3,3',4',5,6,7,8-heptamethoxyflavone, NTD; natsudaidain, TNG; tangeretin.

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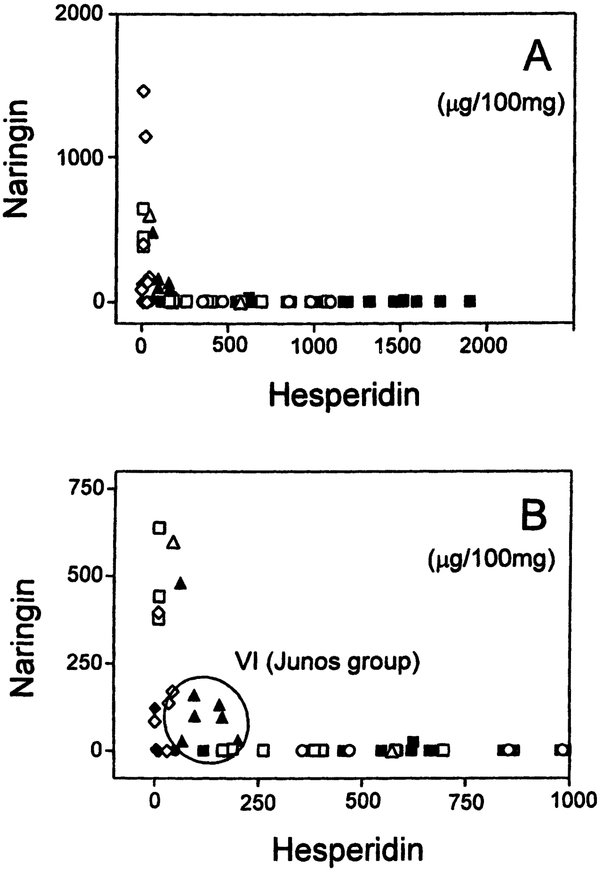

***Figure 2.** Exclusive relationship between hesperidin and naringin contents. (A)Projection of scatter diagram of hesperidin versus naringin, and (B) zoom of the plot of hesperidin versus naringin. Symbols; △ group II; O, group III; , group IV; □, group V; , group VI; ■, group VII; ●, group VIII; ◆, kumquats and Trifoliate orange according to Tanaka's classification. Open symbols indicate Archicitrus and closed symbols for Metacitrus.*

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Table II. HL-60 Differentiating Activity of Representative Citrus Juices

Conventional name	Scientific name	Yield (mg)	NBT reducing cell (%)	Total activity
Tahiti lime	*C. latifolia*	1203	20	
Sweet limee	*C. limettioides*	909	45	4545
Bergamot	*C. bergamia*	1024	60	5120
Eureka lemon	*C. limon*	1178	10	
Hirado	*C. grandis*	525	21	
Marsh grapefruit	*C. paradisi*	977	14	
Red blush	*C. paradisi*	1829	15	
Kinukawa	*C. glabeerrima*	241	21	
Hassaku	*C. hassaku*	628	14	
Natsudaidai	*C. natsudaidai*	1199	33	
Sanbokan	*C. sulcata*	620	20	
Sour orange	*C. aurantium*	898	14	
Valencia	*C. sinensis*	611	20	
Morita navel	*C. sinensis*	565	12	
Oto Mikan	*C. sinograndis*	550	14	
Iyo	*C. iyo*	607	15	
Hyuganatsu	*C. tamurana*	1074	15	
Yuzu	*C. junos*	1023	18	
Kunenboo	*C. nobilis*	415	13	
King	*C. nobilis*	1172	67	5860
Sugiyama Unshiu	*C. unshu*	520	33	
Okitsu Wase	*C. unshu*	172	17	
Yatsushiro	*C. yatsushiro*	541	18	
Kabuchi	*C. keraji*	375	11	
Ota Ponkan	*C. reticulata*	510	11	
Dancy tangerin	*C. tangerina*	180	21	
Clementine	*C. cleentina*	587	17	
Jimikan	*C. succosa*	1473	14	
Shikaikan	*C. suhuiensis*	582	15	
Kobeni Mikan	*C. erythrosa*	231	50	1157
Hirakishu	*C. kinokuni*	637	15	
Shiikuwasha	*C. depressa*	459	54	2293
Koji	*C. leiocarpa*	369	21	
Shikikitsu	*C. madurensis*	1012	11	

The classification and the nomenclature of Citrus plants were based on Tanaka's classification. Yield from 100 mL of juice. HL-60 differentiation assay was done at a concentration of 200 mg/mL. Total activity of the juice was calculated from a division of the fraction yield by the concentration examined for HL-60 differentiation activity (200 µg/mL), and when > 40% of HL-60 cells were induced to have NBT reducing activity, a sample was judged as active.

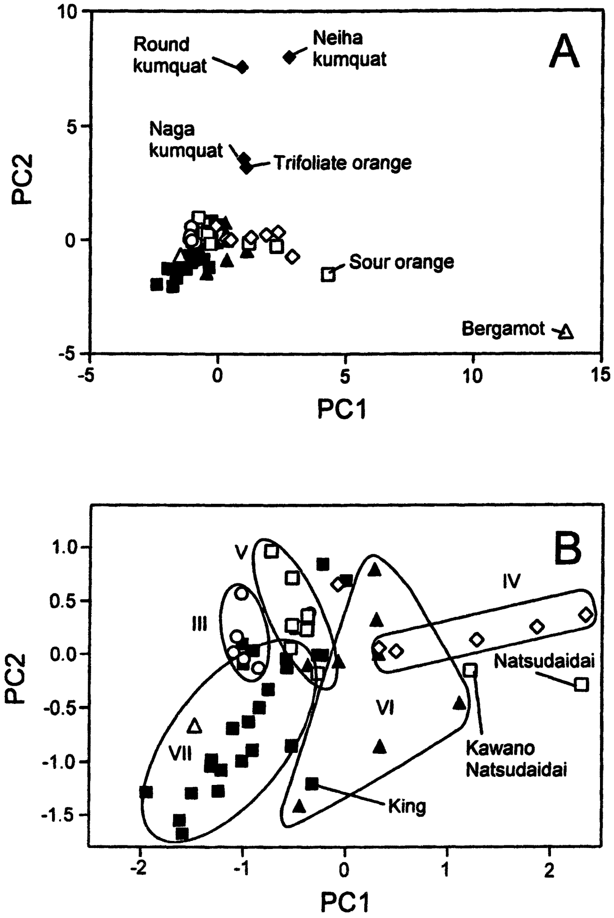

Figure 3. (A)Projection of scatter diagram from a principal component analysis, PC1 versus PC2, and (B) zoom of the plot of PC1 versus PC2. Symbols; △ group II; ○, group III; , group IV; □, group V; , group VI; ■, group VII; ●, group VIII; ◆, kumquats and Trifoliate orange according to Tanaka's classification. Open symbols indicate Archicitrus and closed symbols for Metacitrus.

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The Tanaka's classification system was an excellent descriptive morphology of *Citrus* biotype, and equates morphology diversity with speciation. Tanaka classified the forms of Citrus into two subgenera, namely Archicitrus and Metacitrus subgenera, based on their inflorescence pattern, eight sections, thirteen subsections, eight groups, and 145 species (*15*). Quantitation of flavonoid in the edible part of various *Citrus* fruits suggested that flavonoid content followed the variation of morphological *Citrus* biotypes, since some agreement with the Tanaka's classification system was observed.

Factor loadings indicated the relative extent to which each original variable contributes to the variance contained in each principal component. PC3 and PC5 strongly correlated with nobiletin and tangeretin, and heptamethoxyflavone and natsudaidain, respectively. Similar factor loadings of nobiletin and tangeretin for PC3 (0.35 and 0.34, respectively) and the coefficient of correlation (r = 0.9075) between these polymethoxyflavone contents suggested possible relationship between them. Similar relationship was also suggested for heptamethoxyflavone and natsudaidain (factor loadings are -0.54 and -0.60 for PC5, respectively, r = 0.4810). This significance of correlation between nobiletin and tangeretin, and between heptamethoxyflavone and natsudaidain suggested existence of a common biosynthetic pathway. Tangeretin has a similar structure as nobiletin, but it lacks C-3' methoxyl group of nobiletin. Heptamethoxyflavone is a 3-OH methylated derivative of natsudaidain. These structural similarities also support the above-mentioned suggestion.

Scatter diagram for nobiletin and natsudaidain (Figure 4) demonstrated peculiarities of King, Koji (*C. leiocarpa*), Oto Mikan (*C. sinograndis*), Ichang lemon (*C. wilsonii*), Kourai Tachibana (*C. nippokorean*), Shiikuwasha, and Ponkan (*C. reticulata*). Especially, King, Koji, and Oto Mikan demonstrate characteristic pattern, because these species contained a higher concentration of heptamethoxyflavone and natsudaidain than those of nobiletin and tangeretin whereas other species showed a reverse profile of polymethoxyflavone contents. Characterization of polymethoxyflavones in hybrids of the high nobiletin-tangeretin species and the high heptamethoxyflavone-natsudaidain species will give us important information on inheritance of polymethoxyflavone.

CONCLUSIONS

The aim of this study was to provide a better understanding of genetic relationships in Citrus and related genera by flavonoid profile. The HPLC analysis of 24 flavonoids in edible parts was carried out on representative or economically important *Citrus* species, cultivar, and near citrus relatives. Our

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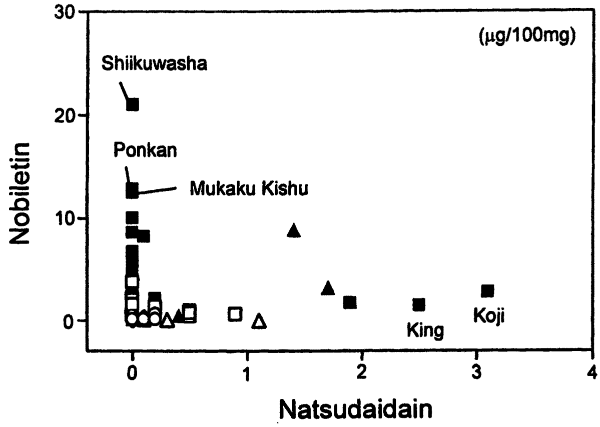

Figure 4. Relationship between nobiletin and natsudaidain contents. Symbols; △ group II; O, group III; , group IV; ☐, group V; , group VI; ■, group VII; ●, group VIII; ◆, kumquats and Trifoliate orange according to Tanaka's classification. Open symbols indicate Archicitrus and closed symbols for Metacitrus.

results suggested that polymethoxyflavones in *Citrus* juices are important for their anti-cancer action. We precisely evaluated the influence of cultivar on flavonoid composition by multivariate analysis. This flavonoid survey on both commercial cultivars and important hybrids, will aid not only in taxonomic evaluation by genetics and inheritance patterns, but also in horticultural breeding programs focusing on health promotion by selecting varieties rich in anticancer substances.

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Chapter 8

Antioxidant Activity of Anthocyanins In Vitro and In Vivo

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Diets rich in antioxidants may decrease the risk of developing major diseases. Intakes of anthocyanins, polyphenolic secondary plant metabolites responsible for the red and purple colors of soft fruits and certain vegetables, may exceed 200mg/day but little is known about their ability to act as antioxidants. Application of electron spin resonance spectroscopy to 13 anthocyanins illustrated the importance of the degree of hydroxylation, glycosylation and pH in determining antioxidant capacity. Although consumption of anthocyanin-rich extracts decrease DNA damage and indices of lipid peroxidation in rats with a compromised antioxidant status, it is not yet clear whether anthocyanins function as antioxidants at nutritionally-relevant dietary intakes.

Introduction

Several epidemiological studies suggest that diets rich in polyphenolic products of the phenylpropanoid biosynthetic pathway in plants decrease the risk of developing major diseases including heart disease and certain cancers (*1*). Such potential health effects may be a reflection of the ability of many polyphenols to prevent the oxidation *in vitro* of biological molecules such as proteins, lipids and DNA (*2*). Such antioxidant effects are mainly due to the ease with which an H-atom from an aromatic hydroxyl group of polyphenols can be

In Nutraceutical Beverages; Shahidi, F., et al.;
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donated to a free radical and the ability of the aromatic group to then support the unpaired electron via delocalisation around the π-electron system (*3*). Countries in Northern latitudes which generally have the greatest number of premature deaths from chronic diseases also tend to have a habitually low consumption of foods rich in phytochemicals with antioxidant activity (*4*). This may be due to traditional dietary patterns and the expense and lack of availability of fresh fruits and vegetables. For such populations, a potentially important source of antioxidant-rich food may be locally grown soft fruits (eg. raspberries, blackberries, blueberries, cranberries and blackcurrants). These are rich in anthocyanins (Figure 1), which are glycosidic-linked flavonoids responsible for the red, violet, purple and blue colors of many plants (*5*). Anthocyanins are also increasingly used as food colorants. Consequently, in this chapter we discuss relevant data assessing the ability of anthocyanins to act as antioxidants in chemical and biological systems.

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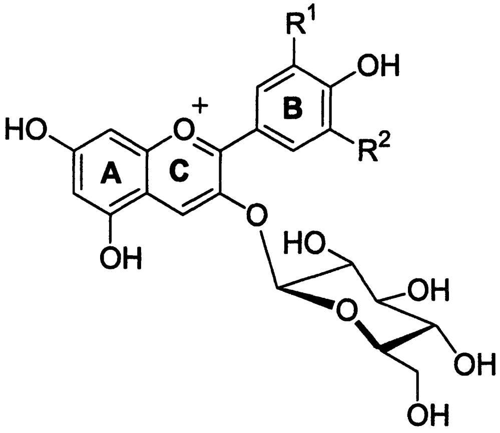

	R^1	R^2
Pg-3-glc	H	H
Mv-3-glc	OMe	OMe
Pn-3-glc	OMe	H
Pt-3-glc	OMe	OH
Cy-3-glc	OH	H
Dp-3-glc	OH	OH

Figure 1. The structures of some anthocyanin glycosides: Pg – pelargonidin; Mv – malvidin; Pn – peonidin; Pt – petunidin; Cy – cyanidin; Dp – delphinidin. The anthocyanidin aglycones have OH at the 3 position.

Antioxidant capacity of anthocyanins *in vitro*

The antioxidant potential of anthocyanins were determined by their abilities to reduce stable radical species by hydrogen atom or electron transfer reactions. In organic media, galvinoxyl radical (2,6-di-*tert*-butyl-α-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyloxy) was used. This is a resonance-stabilised, sterically-protected, phenoxyl radical which dissolves in a wide range of organic solvents, including ethanol, and has a half-life of several hours. Consequently, it may be a useful determinant of relative activities in the aprotic environment of the lipid phase of foods and in cell membranes. Fremy's radical (potassium notrosodisulfonate), which is highly water soluble, can give complementary information on relative activities in the aqueous environment. Both radicals have well-defined electron spin resonance (ESR) spectra which can be monitored to assess the extent of reduction in the presence of antioxidant functionals thereby allowing the reaction stoichiometry to be determined. Furthermore, they are not sufficiently oxidising to indiscriminately abstract hydrogen atoms from a wide range of biological substrates and thus react preferentially with compounds which may fulfill an antioxidant role *in-vivo*. In comparison with coulorimetric methods, ESR detection has distinct advantages in terms of sensitivity, lack of interference with the highly-resolved and unique radical spectra, and the ability to work with strongly-colored or turbid solutions. Full details of the method are described in Garder *et al.* (*6*).

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Reaction stoichiometry

Using ESR, many anthocyanins were found to possess antioxidant activity similar to vitamin E (dα-tocopherol) and its synthetic water-soluble derivative, Trolox (Figure 2). For example, one molecule of the aglycones of cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (i.e. the anthocyanidins) is capable of reducing more than one molecule of galvinoxyl radical, stoichiometries ranging from 1.0 to 2.2. These compare with stochiometries of 2.2 and 2.3 for dα-tocopherol and Trolox, respectively. In aqueous medium, using Fremy's radical, reaction stoichiometries ranged from 0.4 to 1.6. Structure-activity relationships were less evident than has been found previously with flavonoids where the extent of B- and C-ring hydroxylation impacted markedly on activity (*6*). Moreover, unlike flavonoids, substitution of the C-ring at the 3 position with a glycoside unit did not adversely affect reactivity. In fact, glycosylation generally increased antioxidant capacity although the mechanism behind this phenomenon is still unclear.

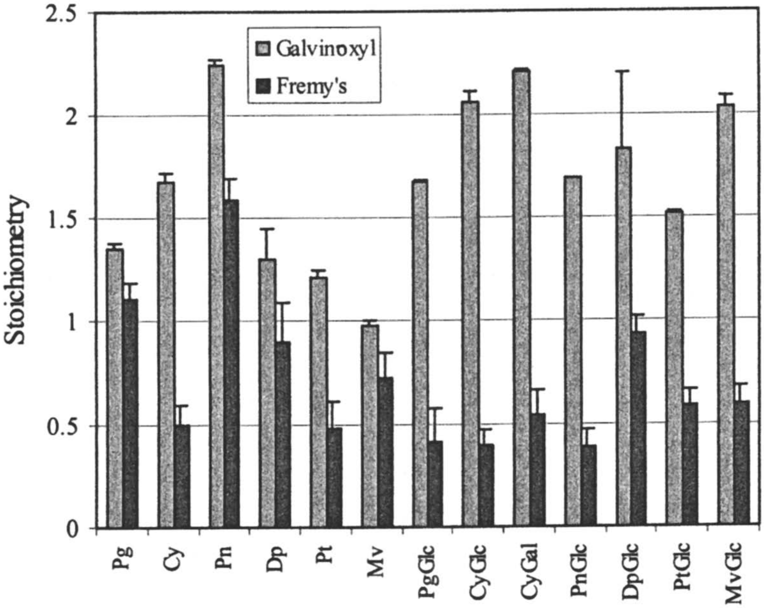

Figure 2. Antioxidant capacity of anthocyanins. Pg: Pelargonidin; Cy: Cyanidin; Pn: Peonidin; Dp: Delphinidin; Pt: Petunidin; Mv: Malvidin; PgGlc: Pelargonidin 3-glucoside; CyGlc: Cyanidin3-glucoside; CyGal: Cyanidin 3-galactoside; PnGlc: Peonidin 3-glucoside;DpGlc: Delphinidin 3-glucoside; PtGlc: Petunidin 3-glucoside; MvGlc: Malvidin 3-glucoside

Effects of pH on antioxidant capacity

Unlike other products of the phenylpropanoid pathway, anthocyanins are charged species whose chemical structure changes with alterations in pH (*7*). At low pH, they exist predominantly as the flavylium cation. At pH 5.0, a colorless carbinol is formed and as the pH becomes alkaline, equilibrium exists between the quinoidal pseudobase form and what may be an anionic form of the flavylium structure (Figure 3). Such changes in structure can be interpolated from the differences obtained in absorption spectra between 350 and 700 nm. For example, when 0.1mM solution of cyanidin 3-glucoside was prepared over a wide pH range using citric acid – Na_2HPO_4 buffer (pH 2.6, 5.0, 7.4) and Tris -

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HCL buffer (pH, 8.9), there was a single distinct peak at pH 2.6 and no absorption at pH 5.0 (Figure 4). The main peak in these spectra are likely to be the blue quinoidal base while the structure causing absorption between 400 and 450 nm may be an anionic form of the flavylium structure.

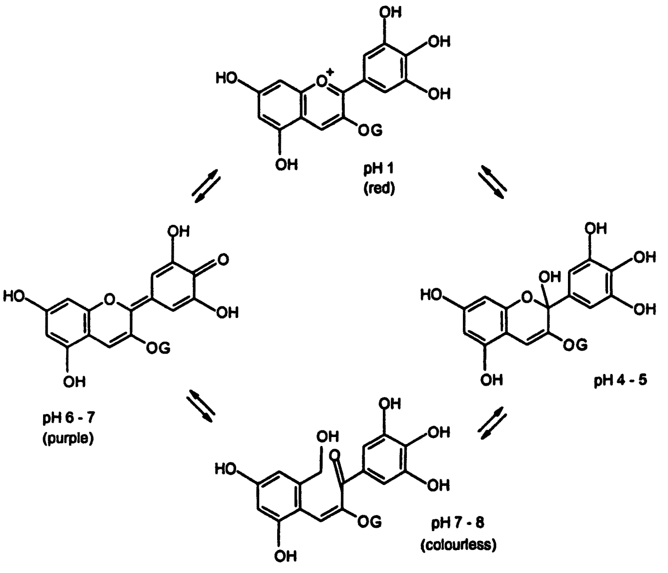

Figure 3: Changes in structure of delphinidin 3-glucoside due to pH (7)

Clearly, as intestinal pH ranges from 2 to 8, structural changes of anthocyanins during transportation through the stomach, small intestine and colon may affect their chemical properties and thus any potential biological effects. For example, the antioxidant capacity of fresh solutions of cyanidin 3-glucoside increases with pH, more Fremy's radicals being reduced in alkaline conditions (Figure 5). This suggests that the flavylium cation is the least active form. At pH 5.0, the carbinol form has a hydroxyl group attached at the 2-C position, creating a chiral center, which may be capable of donating a hydrogen atom to a radical species. In addition, the quionoidal structure formed on the A-ring at alkaline pH may prime the hydroxyl group on the 5-C for hydrogen donation. Therefore, anthocyanins may be more effective as antioxidants in the small intestine and colon compared with the stomach. However, the presence of oxygen causes a decrease in antioxidant capacity at the higher pH values. This may reflect an autoxidation process, oxidation of the carbinol structure producing a colorless chalcone. The "broken" B ring of this molecule would lead to a decrease in antioxidant potential.

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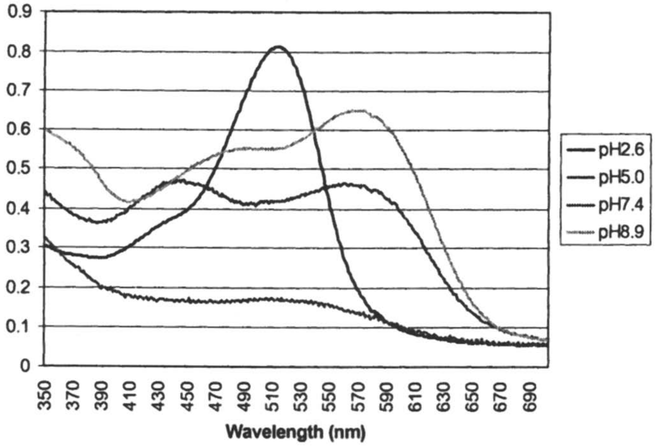

Figure 4. Absorption spectra of cyanidin 3-glucoside at various pH values.

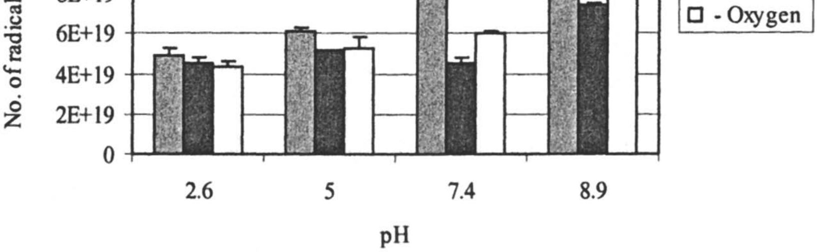

Figure 5. The effect of pH on antioxidant activity of cyanidin 3 glucoside

Stability and partitioning characteristics of anthocyanins

Synthetic phenolics are widely used by the food industry to delay rancidity and extend the shelf life of food products. However, concern about the safety of synthetic antioxidants has led to increasing interest in naturally-occurring alternatives as these may not only retard rancidity but may also have added health benefits. Ease of use of natural polyphenols in food formulation will depend on a number of factors including their relative stability and their ability to partition into the lipid phases of food matrices.

Stability of solvated anthocyanins

As stability is a major consideration for the inclusion of antioxidants in food processing, we assessed how storage in ethanol over 7 days at 25°C affected concentrations of a range of anthocyanins. Stability was estimated using an adaptation of the method of Ribreau-Gaynor and Stonestreet (*8*) in which absorbances at 520 and 700 nm are measured at pH 0.6 and 3.5. Concentration was calculated from the expression:

$$A_{total} = [A_{520} - A_{700}]_{pH\ 0.6} - [A_{520} - A_{700}]_{pH\ 3.5}$$

Results (Table I) indicate that delphinidin degrades rapidly with time with little remaining after 7 days. Petunidin and malvidin also showed significant loss over this period of 23 and 13%, respectively. However, the compounds were relatively stable as glycosides.

Theoretical octanol-water partitioning coefficients of anthocyanins

Octanol - water partitioning coefficients (LogP values, where P is the ratio of the concentration of a compound in the octanol phase over that in the aqueous phase) have been widely used to model a chemical's lipophilicity. These values are employed by the pharmaceutical industry as one of many QSAR predictors relating to bioactivity and bioavailability. A number of algorithms have been developed to allow theoretical predictions of LogP using fragmental, atom, neural-network and quantum based approaches of which the fragmental methods appear most robust. Consequently, we have used the ClogP algorithm (*9*) to predict the partitioning coefficients of six anthocyanins in their flavylium form, their 3-glucoside derivatives and the pH-dependent equilibria structures of malvidin.

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Table I. Stability of solvated anthocyanins upon storage

Anthocyanin	*4 days*	*7 days*
Pelargonidin	-3.88	-6.64
Cyanidin	-0.23	-3.02
Peonidin	-4.26	-6.50
Delphinidin	-75.15	-94.48
Petunidin	-11.50	-12.78
Malvidin	-16.54	-22.90
Pelargonidin 3-glucoside	-8.08	-4.37
Cyanidin3-glucoside	0.48	0.32
Cyanidin 3-galactoside	-5.85	-2.44
Peonidin 3-glucoside	-0.47	-2.60
Delphinidin 3-glucoside	-2.20	-4.16
Petunidin 3-glucoside	0.23	-0.70
Malvidin 3-glucoside	-1.29	-4.18

NOTE: Data as percentage change

Table II. ClogP values of flavylium aglycones and glycosides

Compound	*ClogP value*
Pelargonidin	2.16
Peonidin	2.02
Malvidin	1.80
Cyanidin	1.56
Petunidin	1.47
Delphinidin	0.897
Pelargonidin 3-glucoside	0.783
Peonidin 3-glucoside	0.636
Malvidin 3-glucoside	0.415
Cyanidin3-glucoside	0.187
Petunidin 3-glucoside	0.090
Delphinidin 3-glucoside	-0.479

NOTE: A value of zero is a prediction of 50:50 octanol – water partitioning.

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The ClogP algorithm predicts that all of the flavylium aglycones will preferentially partition within the lipid environment (Table II).

The values follow the expected trend towards decreasing hydrophobicity as the B-ring becomes more hydroxylated as exemplified by the series: Pelargonidin, cyanidin and delphinidin. Methoxylation has a marginal impact on lowering hydrophobicity (pelargonidin, peonidin and malvidin or cyanidin and petunidin). Accounting for the log scale, delphinidin, although lipophilic, is predicted to have a significant presence (13%) in the aqueous phase. Introduction of the 3-glucoside unit is predicted to dramatically decrease hydrophobicity. Pelargonidin, the most lipophilic anthocyanin, when glycosylated becomes less hydrophobic than all other aglycones. Petunidin-3-glucoside is predicted to partition almost equally between both phases, whilst delphinidin-3-glucoside acquires significant lipophobic character with almost 90% distributed in the aqueous phase.

The potential effects of pH on partitioning are predicted by calculations on the various forms of malvidin that have been reported (*7*) to exist in a series of pH-dependent equlibria (Figure 6).

The flavylium cation, which dominates at low pH, is highly lipophilic with only 1.6% predicted to be present in the aqueous phase. However, structures existing in the physiological pH range are much less hydrophobic with ClogP values approaching 0.5, which equates to approximately 30% presence in the aqueous phase. Although the ClogP algorithm has a high predictive accuracy with R = 0.936 when compared against experimental values from a large drug dataset, it must be emphasised that if structural attributes are present within the anthocyanins which are not well-defined within the algorithm's training set of over 1000 compounds, then the predictive accuracy may be lower.

Nutritional relevance of anthocyanins

Epidemiological studies inversely relating dietary intakes of plant polyphenols with the incidence of heart disease and cancer (*10*) may indicate a putative role for anthocyanins in the prevention of chronic diseases. To date, formulation of databases to allow estimates of dietary polyphenol intakes have mainly focussed on flavonols and flavones rather than anthocyanins *per se*. There is therefore a need for dietary compositional information to facilitate epidemiological and human intervention studies assessing the role of anthocyanins in health.

Anthocyanins in foods

In the US, average daily intake of anthocyanins may be around 200mg/day (*5*), which is comparable to intakes of recognized nutritional antioxidants such as

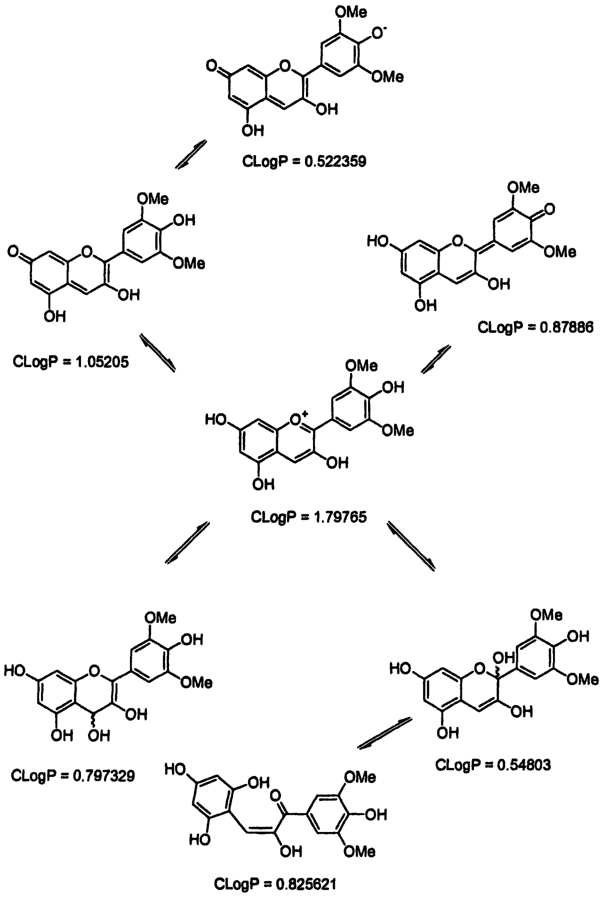

Figure 6. Relationship between ClogP and the various structures which malvidin is reported to form in the literature (7) as a function of pH. The flavylium cation represents the major form at low pH.

vitamin C, vitamin E and certain carotenoids. However, accurate determination of dietary intake of anthocyanins is problematical due to variations in analytical methodology. In addition, the concentration of anthocyanins in foods and beverages can be influenced by several factors including species, variety, light, degree of ripeness, processing and storage. In general, greatest concentrations of anthocyanins are found in soft fruit, significant amounts also being present in some vegetables and beverages (Table III). Red wine is also a rich source of anthocyanins. However, during the maturation process, there is a color change (520nm towards 420nm) reflecting the progressive displacement of the grape

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Table III. Anthocyanin types and contents in selected foods

Food	*Content*[1] *(g/kg)*	*Anthocyanin*[2]	*Glycosides*[3]
Red apple	0.1-0.2	Cy	Glu; gala; arab
Bilberry	3.7	Mv; Dp; Cy; Pt; Pn	Glu; gala; arab
Blackberrys	0.3-1.1	Cy; Pg	Glu; gala; rut; arab; xyl
Blueberry	0.8-2.8	Mv; Cy; Dp; Pn; Pt	Arab; gala; glu; 6-ace-3-gly, sam; soph
Cherry	2.4	Cy; Pn	Glu; rut
Cranberry	0.1-3.6	Pn; Cy	Gala; glu; arab
Current, black	1.3 - 4.0	Cy; Dp	Glu; rut
Elderberry	2.0-10.0	Cy	Sam; glu; sam-5-glu, 3,5-diglu, *p*-cou-glu-5-glu
Red grapes	0.7 – 1.1	Mn; Dp; Pt; Pn; Cy	Glu; glu-ace; glu-*p*-cou; rut
Lychee	0.5	Cy, Mv	Glu, rut, ace-glu
Orange, blood	2	Cy; Dp	Glu; mal-glu
Peach	0-0.1	Cy	Glu; rut
Plum	0.02-0.3	Cy	Glu; rut; 3-ace-glu; gal
Raspberry	0.3-1.2	Cy; Dp; Mv; Pg	3-glu; soph, rut; 3-glu-rut
Strawberry	0.1 – 3.8	Pg; Cy	Rut; 3-ace-gly; glu
Red cabbage	0.7-0.9	Cy	3,5-diglu; soph-5-glu acylated with p-cou, fer, sin
Red onion	0.2	Cy; Pn	Mal-glu; di-mal-lam; mal-lam; glu; mal-3-glu; arab
Red radish	1.5 (skin)	Pg; Cy	3,5-diglu; soph-5-glu acylated with p-cou, fer, caf
Black beans	2.1	Dp; Pt; Mv	Glu
Cocoa beans	0 – 1.0	Cy	Arab; gala; arab-glu
Purple basil	0.2	Cy, Pn	Glu; 3,5-glu; p-cou; mal; p-cou-glu-5-glu
Red wine	0.01-0.5	Mv; Dp; Pt; Pn; Cy	glu; ace-glu; *p*-cou-glu; 3,5-glu
Blackcurrant juice	0.02 – 0.1	Cy	Glu; rut

NOTE: Data from several sources

[1] crude total anthocyanin content

[2] Cy - cyanidin; Dp – delphinidin, Mv – malvidin; Pn – peonidin; Pg – pelaronidin

[3] Glu – glucoside; gala – galactoside; arab – arabinoside; rut – rutinoside; xyl – xyloside; rham – rhamnoside; soph – sophoroside; mal – malonyl; lam – laminaribioside; samb – sambubioside; *p*-cou – *p*-coumaroyl; ace – acetyl; caf – caffeoyl; fer – feruloyl; sin – sinapic

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anthocyanins by more stable polymeric pigments which are formed by a variety of reactions including co-pigmentation (*11*).

Although anthocyanins have marked antioxidant activity in chemical systems, they have to be absorbed from the gut if they are to exert a similar effect in systemic cells and tissues. As yet, little is known about the bioavailabilty of anthocyanins although it will likely be affected by numerous factors including molecular structure, the amount consumed, the food matrix, degree of bioconversion in the gut and tissues, the nutrient status of the host and genetic factors. Direct intestinal absorption of anthocyanins in the intact glycoside form and subsequent biotransformation to methylated derivatives in the liver has been observed in animal models suggesting that some may be bioavailable (*12*). In addition, we have observed that anthocyanin rich-extracts may moderate oxidation *in vivo* (*13*). Rats were maintained on vitamin E-deficient diets for 12 weeks in order to enhance susceptibility to oxidative damage and then repleted with rations containing a highly purified anthocyanin-rich extract at a concentration of 1g/kg diet. The extract consisted of the 3-glucopyranoside forms of delphinidin, cyanidin, petunidin, peonidin and malvidin. Consumption of the anthocyanin-repleted diet significantly decreased the vitamin E deficiency-enhanced hydroperoxides and 8-Oxo-deoxyguanosine concentrations in liver. As these are indices of lipid peroxidation and DNA damage, respectively, dietary consumption of anthocyanin-rich foods may have nutritional benefits. However, it should be noted that in such studies the animals are fed quantities of the compounds, which markedly exceed what may be achievable from diet alone. The nutritional relevance of these studies is therefore uncertain. Clarification of the absorption, bioavailability and metabolism of the anthocyanins in our diet will be an important research area in the future.

Acknowlegements

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Chapter 9

Functional Components in Citrus Beverages

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Citrus beverages are often part of our daily diet. Recent animal, case and cohort control as well as epidemiological studies have shown that certain functional components [carotenoids (lycopene and β–carotene), limonoids, flavonones (naringin and naringin rutinoside), folate, and vitamin C] of citrus prevent chronic diseases such as cancer and cardiovascular diseases. Functional components from three single strength commercial 'Rio Red' grapefruit (*Citrus paradisi* Macf.) juice products namely cartons (100% pure fresh juice and not from concentrate), cans (100% juice from concentrate), and cocktails (35% juice from concentrate), were analyzed by reverse phase liquid chromatography. The levels of vitamin C significantly decreased when half-gallon grapefruit juice cartons were stored at 4°C for six weeks, but the levels of limonin 17-β-D glucopyranoside (LG) were not affected during the same period. Total flavonone and lycopene levels were significantly higher at six weeks compared to initial levels. Although a significant loss of vitamin C levels was found when cans were stored at room temperature (24°C) for 12 months, LG levels were markedly increased during the same period. Furthermore, total flavonone and carotenoid levels were changed during storage, but no specific trend was observed. When cocktail in polyethylene toluene (PET) containers was stored at room temperature, the levels of vitamin C were significantly decreased; however, LG and lycopene levels were increased. Our experiments suggest that the functional components in grapefruit juice change during storage, depending on the container used. To remain competitive in international and national markets, optimization of these components may be important for the processing industry.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

The economic value of the fruit juice industry in the world exceeds $10 billion and is one of the largest agricultural businesses in the world (*1*). Quality of processed citrus juice plays an important role in consumer acceptance and its overall success in the marketplace. During the last decade, juice quality may not only be restricted to changes in soluble solids, acidity and their ratios during storage but may also depend on changes in levels of functional components of citrus including carotenoids (lycopene and β–carotene), limonoids, flavonones (naringin and naringin rutinoside), folate, vitamin C, and coumarins. Citrus processed products are considered the best sources of natural vitamin C, therefore its stability during storage is crucial for the viability of juice industry (*2*). Retention of certain minerals may be possible by grapefruit juice. For example, Mallon and Rosenblatt (*3*) demonstrated that 5 ml of grapefruit juice daily but not tomato juice increased calcium retention in female rats from 89.9 to 96.9 %. In order to retain quality components of juice, several packaging materials are used which include glass, polyethylene, polystyrene, cardboard, plastic and foil laminates, as well as aluminium and tin coated steel cans for various citrus products (*4,5*).

Low temperature storage of canned juices may be beneficial to the retention of functional components in several ways. In general, the loss of vitamin C is minimal at storage temperatures of 5^0C or less; however, when juice is stored at 25-30^0C for a period of 12 months, loss of vitamin C is approximately one-quarter of its original content (*6*). It has also been shown that carotenes in canned juice are more stable than vitamin C (*6*).

While the the type of storage container has significant effects on vitamin C when stored for several months (*7*), no marked differences in vitamin C content was observed when stored for a short period (*8, 9*). In general, storage time may be more important in the loss of vitamin C than type of storage container (*10*).

Although several studies have been conducted in relation to the stability of vitamin C, its influence on quality during storage, and its influence on processing (*11, 12*), very little information is available on the effect of storage and processing on stability of other functional components in citrus (carotenoids, flavonones, and limonoids). Neverthless, several studies have been conducted on carotenoids such as β-carotene and lycopene changes in vegetables during storage. Changes in β-carotene during storage and processing of vegetables have been studied by several authors but results show no definite trend of β-carotene retention. Reports on the stability of β-carotene range from no loss to

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slight to marked decrease when total carotenoids were measured (*13, 14*). Recent studies have demonstrated that β-carotene remained stable in both refrigerated and frozen broccoli during storage (*15*).

Cumulative evidence suggest that fruits and vegetables protect against cancer (*16, 17*) and this has stimulated research on elucidiating the responsible biological mechanisms. Both in *vitro* and *in vivo* studies in the past decade have demonstrated that synergistic effects of several functional components, and/or individual components, have the potential to prevent cancer and chronic diseases. Understanding the bioavailabilty of functional components is critical to elucidiate their potential protection benefits in relation to cardiovascular, cancer and other chronic diseases. Until these questions are answered, it would be difficult to gain further insights into the biological activities of functional components.

By virtue of its deep red color, sweet flavor, texture and quality of the fruit, consumer acceptance of the Rio Red grapefruit in the market has been increasing at a rapid rate. Despite the potential benefits of citrus consumption to human health, consumption of grapefruit juice while taking certain medications has been restricted because grapefruit juice has been shown to elevate the pharmacological efficacy of orally administered drugs such as felodipine, nisoldipine, cyclosporin, terfenadine, Midazolam, and lovastatin, and sometimes develop side reactions through yielding conditions with extraordinary high plasma concentration. Bioavailibility of these drugs after oral administration are increased 1.5 to 15 fold by the ingestion of grapefruit juice (*17, 18, 19*). While studies are in progress to understand the role of the specific component which interacts with these drugs, it is very important to determine changes in functional components during storage.

Materials and Methods

Sources of samples

Three single-strength commercial 'Rio Red' grapefruit (*Citrus paradisi* Macf.) juice products, namely cartons (100% pure fresh juice and not from concentrate), cans (10% juice from concentrate), and cocktails (35% juice from concentrate), were obtained from Texas Citrus Exchange, Mission, TX, USA.

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Storage Conditions

A total of 60 half-gallon paper cartons were stored in a refrigerator (4^0C) for six weeks simulating home conditions and every two weeks five cartons were used for each component analysis. Four hundred and four 177 mL cans of grapefruit juice from concentrate were stored at room temperature. Each month 10 cans for each component were analyzed separately for 12 months except carotenoids which were analyzed for only seven months. Forty-eight polyethylene toluene (PET) 1.86 L containers of cocktail were stored at room temperature (~24 C) and every month three bottles were analyzed for each component. Filtered samples were collected in 50 mL tubes and stored in –80°C until samples were analyzed for limonoids, flavonones and vitamin C but samples for carotenoids were extracted in hexane immediately and stored at -80^0C.

Chromatographic conditions

High performance liquid chromatography (HPLC) system consisted of a Perkin-Elmer binary LC pump 250, an LC-600 autosampler, a UV/VIS Spectrometric detector LC295, and PE 1020 Integrator program, was used for limonoid and flavonone analysis. The same HPLC system with an HP 3394 integrator was used for measuring total vitamin C.

Limonoid analysis

Juice samples were filtered through qualitative P8 filter paper (Fisher Scientific, Pittsburg, PA). The limonin 17-β-D-glucopyranoside (LG) was analyzed with little modification from the previous procedure (*21*). Ten milliliters of juice were passed through a C-18 Sep-Pak (Walters Associates, Miltford, MA). LG was retained on the column, rinsed with water, eluted with MeOH and determined by HPLC using a Waters Spherisorb ODS2 (Walters Associates, Miltford, MA) C-18 column (250 X 4.6 mm, 5μm) along with a guard column. One milliliter of juice was diluted with 3 mL of 80% ethanol. The mobile phase consisted of a linear gradient starting with 10 % acetonitrile, 3 mM phosphoric acid and ending at 27% acetonitrile in 5 mM phosphoric acid for 60 min. The flow rate was 1 mL min^{-1}. The detector was set to 210 nm and the LG peak was identified at 21 min.

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Flavonoid analysis

Samples were analyzed for flavonone content by reverse phase HPLC with some modifications of the method reported previously (*31*). An aliquot of juice sample was diluted with dimethyl sulfoxide (1:1, v/v), centrifuged and filtered through 0.45-μm nylon filters (Alltech Associates, Deerfield, IL) and 20 μl of this solution were injected into HPLC system. The same HPLC system as explained in the LG section, was used, and an Adsorbosil C-18 column (250 X 4.6mm, 5μm) was eluted with a linear gradient starting with 10 % acetonitrile in 5 mM phosphoric acid and ending at 26% acetonitrile in 5 mM phosphoric acid for 36 min. The narirutin and naringin peaks were detected at 280 nm with retention times of 25 and 27 min, respectively. The flavonones were identified by confirmation of their respective spectra, authentic standard and retention times.

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Vitamin C

Three milliliters of 3% citric acid were added to 1 mL of juice. The sample was mixed and a 0.8 ml aliquot was centrifuged at 12,512 g for 20 min, and filtered through 0.45-μm nylon 66 filters (Alltech Associates, Deerfield, IL). A 20 μL aliquot of this solution was injected into the HPLC system. A Waters Bondapak (Walters Associates, Miltford, MA) C-18 column (30 X 0.4 cm) with a guard column was used and the flow rate was 1.5 ml. min^{-1}. The mobile phase was acetonitrile: water (70:30, v/v) with 0.01 M ammonium dihydrogen phosphate. Vitamin C was detected at 255 nm and was eluted at 6 min after injection.

Carotenoids

Sample preparation procedure established by previous authors (*22, 23*) was used with minor modifications. Approximately 12 g of pulp sample were weighed in a brown 125-mL Erlenmeyer flask. A 100 mL solvent with hexane: ethanol: acetone (50:25:25, v/v/v) was added to the flask and agitated for 10 min on a magnetic stirrer at 8 speed (Fisher, Pittsburg, PA), 15 ml water were added followed by another 5 min agitation. Solvent layers were allowed to separate into distinct polar and non polar layers. The top hexane layer of 2 mL was taken and dried with liquid nitrogen and heated at 30°C for 5 min. Samples were dissolved in 100 μL tetrahydrofuran (THF), diluted to 2 ml with methanol : THF (75:25), and filtered through 0.45 μl filter prior to injection onto the HPLC system.

External standards were used to quantify the compounds. Peak areas were normalized to the external standard and the standard curve was fitted by linear regression (peak area vs. concentration in $\mu g.g^{-1}$). Total flavonone, concentration was calculated by combining naringin and narirutin. Similarily, total carotenoid concentrations were obtained by combining lycopene and β-carotene.

Statistical analysis

Data were analyzed using a randomized block design using GLM procedures (*24*). Fisher protected LSD was employed for all mean separation analysis.

Results and Discussion

Traditionally, consumer preference for citrus juice has depended on its flavor and the type of container in which the juice is stored. Recently, consumer trend has been changing towards, not only for juice with good flavor, but also for juice with higher levels of functional components. Consumers are interested in drinking juice which may provide potential health benefits, provided credible evidence is established. For example, cumulative epidemiological evidence (*17*) attributed citrus consumption to a significant reduction in risk for a wide variety of cancers in humans (oral cavity, lung, larynx, stomach, esophagus, pancreas, colon, and rectum).

The slogan "Citrus beyond vitamin C" may play a key role in the future of the citrus industry because of cumulative evidence of potential benefits of other nutrient and non-nutrient substances in human health. Historically, vitamin C has been considered as one of the most important essential nutrients in our diet since the discovery in 1907 that prevents scurvy (*25*). In addition, vitamin C has several important functions in human bodies in the synthesis of amino acids, lipids and chlesterol, collagen, wound healing, metabolism of iron etc. Along with its antioxidant potential, vitamin C prevents the inhibition of gap-junction intercellular communication induced by hydrogen peroxide. However, recent report suggests that the flavonol, quercetin, has stronger anticancer activity than vitamin C (*25*).

In citrus, limonoids are formed from aglycones by the addition of one molecule of glucose at the C-17 position in the D ring (*26, 27*). Unlike some of the aglycones, including limonin, nomilin, and obacunone, which are bitter, the limonoid glucosides are tasteless (*27, 28*). Most of the aglycones are nonpolar, while the limonoid glucosides are water-soluble (*26, 27, 28*). Another factor is human consumption. The concentration of mixed limonoid glucosides in orange

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juice is 300-350 ppm. By comparison, the concentration of vitamin C is less than 300 ppm and the concentration of the aglycones is usually less than 6 ppm (*29*).

Accumulative scientific evidence and industry focus have led several companies to initiate work on product development with citrus limonoids. The predominant limonoid glucoside in citrus juice is limonin 17-β-D-glucopyranoside (LG) accounting for 30-60% of the mixture (*30*) and several studies in different laboratories have shown potential health benefits of limonoid glucosides through cell culture and animal studies in relation reduction of oral cancer, breast cancer and cholesterol (*30-33*). These companies are primarily concentrated in Japan, Asia, Australia, and Europe. In Japan, scientists have devised a test product, called *LG1000* which contain mixed limonoid glucosides from orange molasses increased approximately 5-fold higher levels of limonoid glucosides (from 220 ppm to 1,000 ppm) by adding to mandarin juice. Results from consumer focus groups have already indicated that this change in product composition had no affect on color, texture, or taste. While attempts to enhance tasteless limonoid glucosides through molecular techniques and mixing several fruit juices are in progress (*34*), researchers have been developing techniques to remove bitter compounds such as limonin and naringin from grapefruit juice (*35, 36*).

During processing, citrus juices undergo heat treatment and pasteurization which may alter flavor depending on whether the juice is prepared from single strength concentrated, pure not from concentrate and percentage of juice. Historically, the main concern about processing in relation to certain functional components, such as carotenoids, has been preventing their losses. Currently, interest has shifted to the effect of processing on preventing not only loss, but also bioavailability of these components. Consumer preference for cartons is highest followed by cocktail and cans. Cartons contain 100% pure juice not from concentrate where as cans and coktail contain juice from concentrate. In general, level of functional components at the time of fruit harvest does not ensure the same level in juices sold in the market because of changes during processing, storage and type of containers used to store juice (*7, 10*).

Changes in functional components of grapefruit juice stored in cartons

The type of container used to store citrus juice infuences concentrations of functional components (*37*). In general, the loss of vitamin C is greater when juice is stored in bottles compared to cans. Currently, grapefruit juice in cartons is available only in certain major grocery stores in Texas. The changes in levels of functional components in grapefruit juice stored in cartons were analyzed at two week intervals for six weeks and the data are presented in Figures 1a, 1b and

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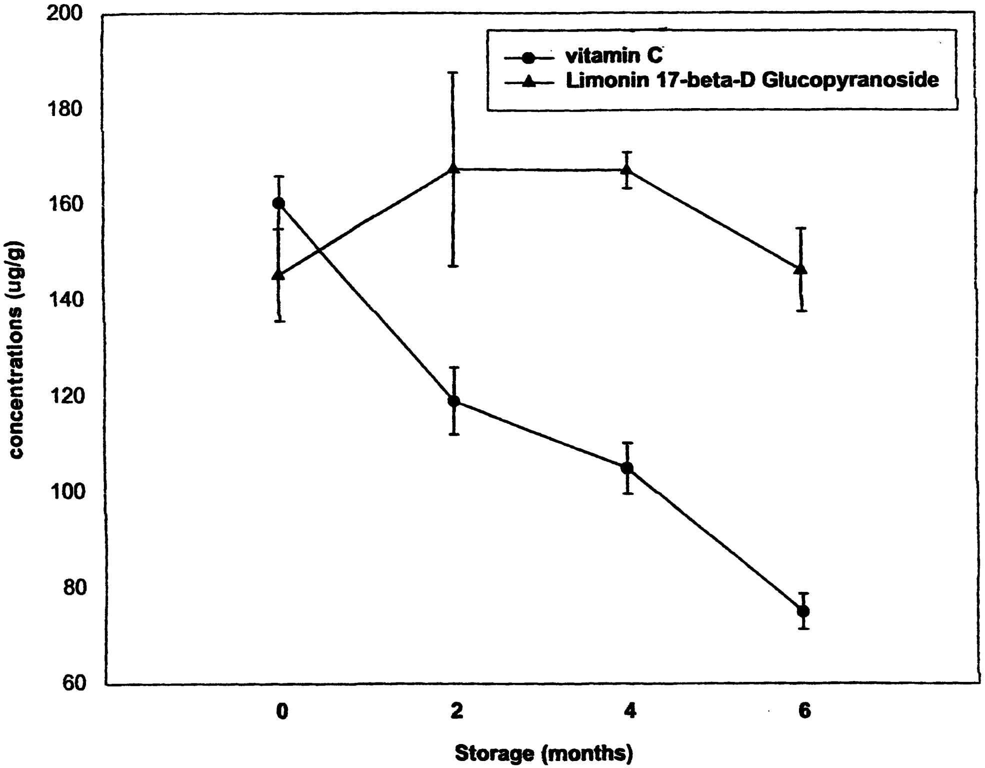

Figure 1a. Changes in limonin 17-β-D glucopyranoside, and vitamin C concentrations during storage of grapefruit juice stored in cartons.

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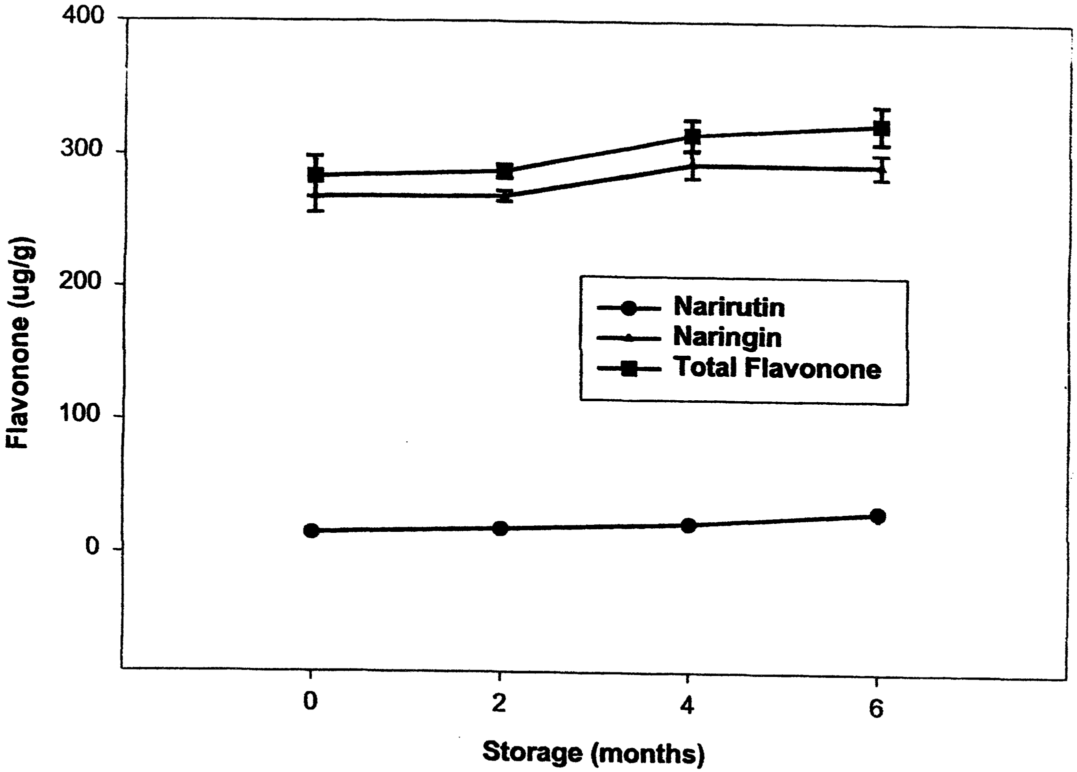

Figure 1b. Changes in naringin, narirutin, and total flavonone concentrations during storage of grapefruit juice stored in cartons.

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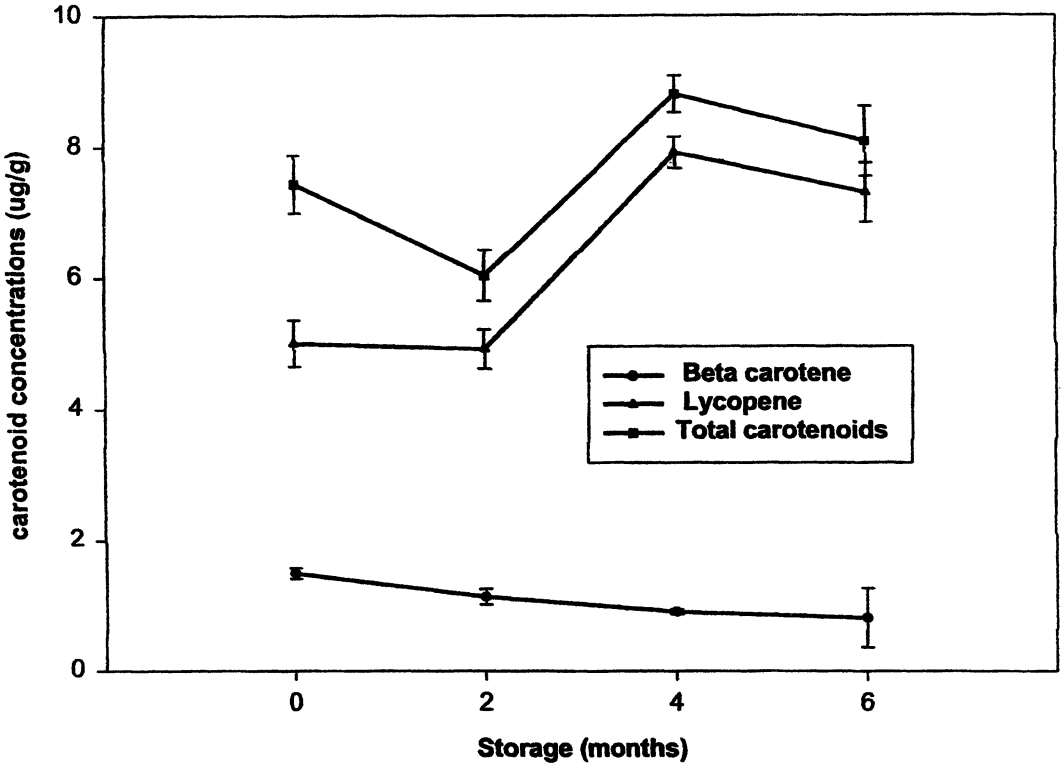

Figure 1c. Changes in β-carotene, lycopene, and total carotenoids concentrations during storage of grapefruit juice stored in cartons.

1c. A significant decrease in vitamin C levels was observed after second and fourth week compared to initial storage. While approximately 54 % of vitamin C was lost during six weeks storage, LG levels were stable during the same period with a slight increase at two and four weeks (Figure 1a). Although narirutin levels were two times higher at six weeks, naringin levels increased slightly (approximately 9%) at six weeks compared to beginning of storage (Figure 1b). Interestingly, 40% increase in lycopene was observed while a significant loss of β-carotene (83%) was measured at the end of six weeks compared to the beginning of storage.

The observed significant loss of vitamin C is consistent with previous results (*6*, *38*). The residual oxygen in the headspace of bottles is attributed to the higher level of degradation of vitamin C, while headspace oxygen is rapidly consumed in corrosion reactions in cans resulting in lower oxidative destruction of vitamin C in cans (*38*). Furthermore, Nagy and Smoot (*39*) have shown that storage temperature in excess of 28^0C caused loss of vitamin C in canned products. This loss was attribuited to oxidation by the residual air layer trapped within the container during processing. The loss was faster in the first two weeks, and was more evident at higher storage temperatures. Nevertheless, when all the residual oxygen is used up, the loss of vitamin C in cans is still possible via anaerobic mechanisms. However previous studies have reported stability of carotenes unlike the increase of lycopene levels observed in our study (*6*).

Changes in functional components of grapefruit juice stored in cans

Changes in levels of functional components in grapefruit juice stored in cans for a year are depicted in Figures 2a, 2b, and 2c. A significant decrease in vitamin C levels was observed every month, and an approximately five-fold decrease in vitamin C was noticed at the end of 12 months. On the other hand, LG levels increased every month, and a two-fold increase in LG was reported at the twelth month compared to the first month. Furthermore, the levels of LG were significantly higher compared to those at intial storage (Figure 2a). Although flavonone concentrations changed slightly during storage, no consistent pattern was observed. Interestingly, no marked changes of naringin and narirutin concentrations at the end of storage was noticed (Figure 2b). Nevertheless, narirutin levels at the sixth month of storage was two-fold higher than at the second month of storage. Unlike other functional components, carotenoid changes were measured for only seven months because of limited samples. Lycopene and β-carotene concentrations decreased significantly after the third month compared to the first month and later the levels increased until seven months (Figure 2c).

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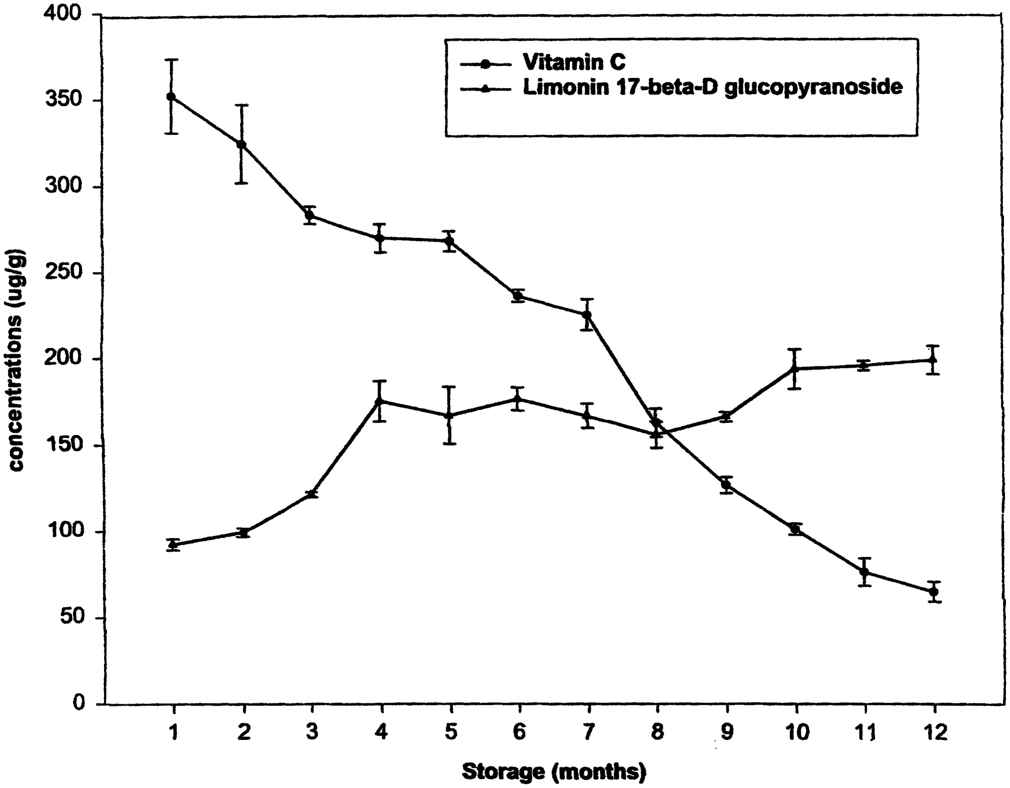

Figure 2a. Changes in limonin 17-β-D glucopyranoside, and vitamin C concentrations during storage of grapefruit juice stored in cans.

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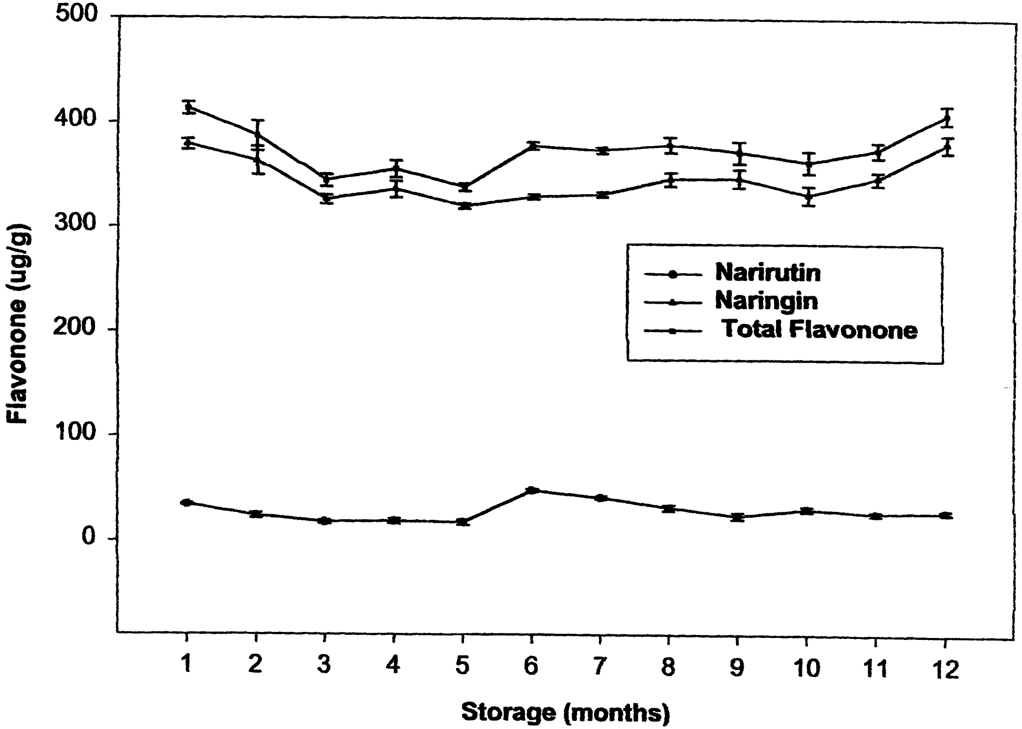

Figure 2b. Changes in naringin, narirutin, and total flavonone concentrations during storage of grapefruit juice stored in cans.

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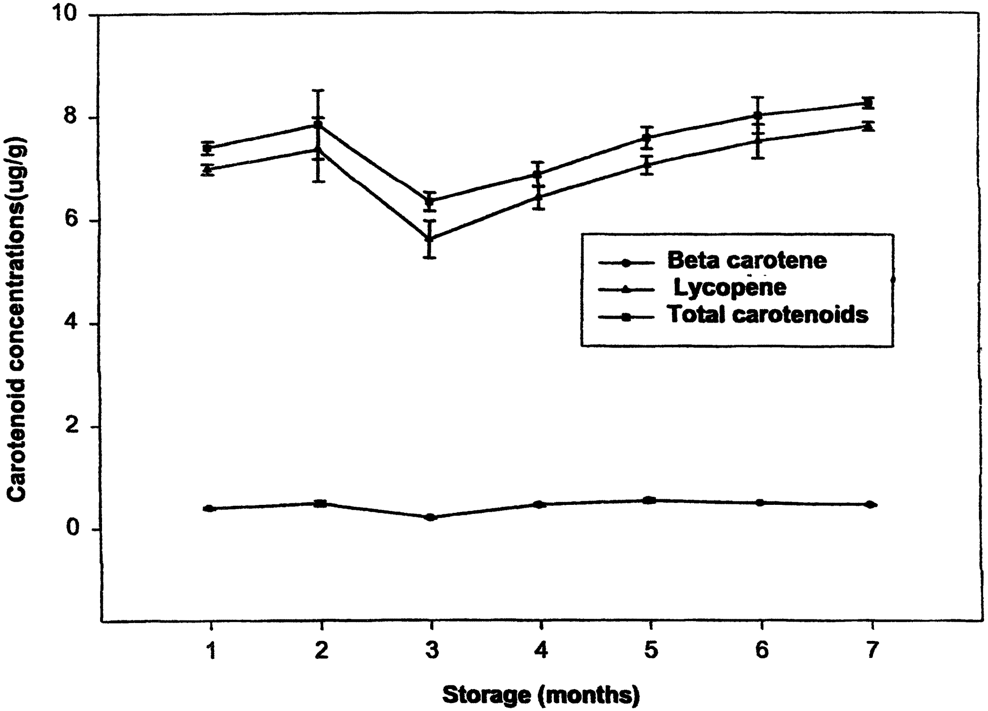

Figure 2c. Changes in β-carotene, lycopene and total carotenoid concentrations during storage of grapefruit juice stored in cans.

Changes in functional components of grapefruit juice cocktail stored in PET containers

Although cocktail contain only 35% of juice from concentrate, people drink it because of its taste, uncloudedness and flavor. Obviously, concentrations of functional components in cocktails stored in PET containers are relatively lower compared to cans and cartons. Approximately, 56 % of vitamin C is lost during four months of storage compared to initial concentrations. A significant decrease in vitamin C was reported immediately after the first month followed by further decreases in subsequent months. A significant increase in LG was found from first to the fourth month, although a slight decrease was noticed at the second month. Although no marked changes in concentrations of lycopene observed during six months of storage, levels of lycopene in the sixth month was significantly higher compared to the second month. (Figure 3a). While naringin concentrations did not alter during storage, narirutin concentrations were significantly different from the beginning of storage, compared to the fourth month, and increased by 29 % in the fourth month of storage (Figure 3b).

Despite the differences in levels of vitamin C at the initial storage in different containers, it is interesting to note that approximately the same level of vitamin C is left at the end of storage in all three container types. Obviously, the concentrations of lycopene in cans and cartons were approximately two fold higher compared to concentrations in the cocktail stored in the PET container. β–carotene was not detected in cocktail. In most cases, levels of flavonones and LG in grapefruit juice and cocktail were similar in all the three containers (Figures 1-3).

It is important to note that level of functional components consumers will attain depends on whether they drink juice or eat the whole fruit. For example, an individual drinking 8 fluid ounce, an amount designated by the Food and Drug Administration, of orange juice will have a daily flavonone intake (as aglycones) in the range 25-60 mg, while those eating a whole 200 g orange will consume 125-375 mg. A single 8 fluid ounce serving of Giant/Minute Maid brand grapefruit juice contains 120 mg naringin and 48 mg narirutin. These values may depend on the cultivar and amount of albedo injested (*40*). An average American daily consumption of grapefruit juice was estimated to be 4.3g (*41*). These values corresponds to an average daily consumption of 2.2 mg naringin and 4.3 mg narirutin, based on values obtained from Giant/Minute Maid juices, respectively (*42*). They are a sizeable fraction of Kuhnau's estimate of 1 g daily intake and surpass the 23-25 mg flavonoid aglcyones per day reported by Hertog and co-workers (*43, 44*).

While the potential benefits of citrus functional components needs further investigation on biological activities in humans, it is important to understand the fact that certain components (carotenoids, flavonones, and limonoids) are either

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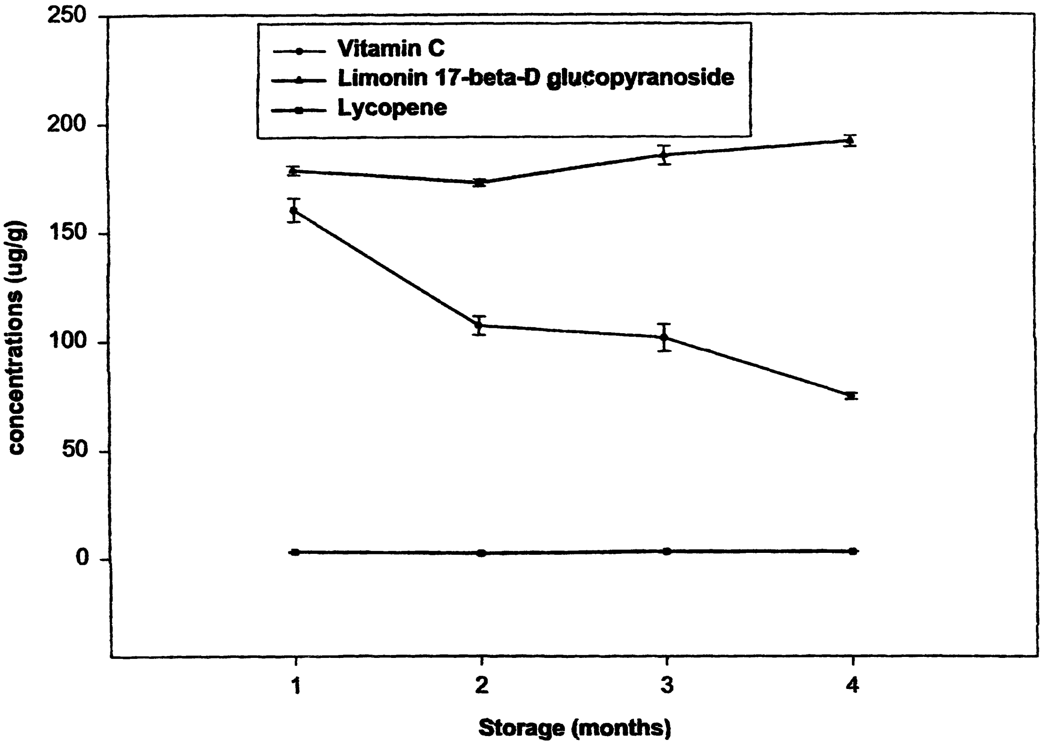

Figure 3a. Changes in limonin 17-β-D glucopyranoside, vitamin C and lycopene concentrations during storage of grapefruit cocktail stored in polyethylene tolune container.

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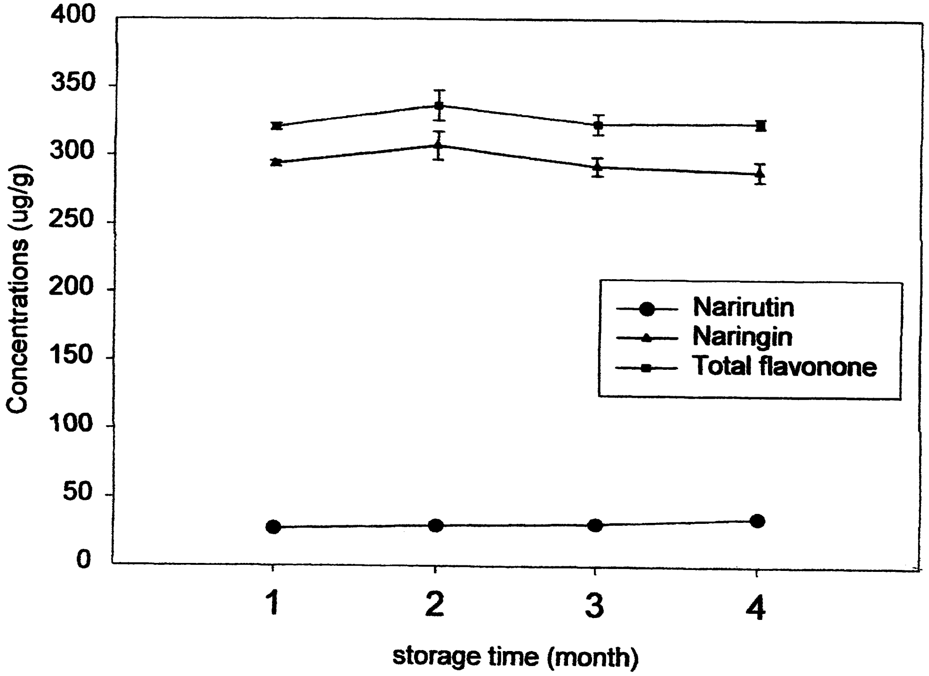

Figure 3b. Changes in naringin, narirutin, and total flavonone concentrations during storage of grapefruit cocktail stored in polyethylene tolune container.

stable or increased, notwithstanding the loss of vitamin C during storage of citrus juice in different containers.

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Chapter 10

Red Berries and Their Health Benefits

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Red berry fruits such as elderberry, chokeberry, and bilberry have a rich history in folk medicine; from cold and flu remedies to diuretic and antirheumatic activities. They are emerging as natural alternatives in modern medicine and as their popularity grows the amount of scientific research conducted on these products also increases. Researchers have studied their health effects in several areas including cardiovascular disease, and as anti-ulcer, anti-viral, anti-inflammatory and stress-reducting activities. The studies described here used varying forms of berries. Many studies describe the anthocyanins as the causative agent responsible for a given activity, but in addition to containing high concentrations of anthocyanins these fruits also contain a complex mixture of other flavonoids. Thus, it is difficult to determine if the anthocyanins are solely responsible for their activities or if anthocyanins are working synergistically with other flavonoids to cause the activity.

In Nutraceutical Beverages; Shahidi, F., et al.;
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Introduction

Red berries have been used in folk medicine for a number of years and now are emerging as a natural alternative in modern medicine. As their popularity grows the amount of scientific research conducted on these products also increases. The red berries discussed here are elderberry (*Sambucus nigra*), chokeberry (*Aronia melanocarpa*), and bilberry (*Vaccinium myrtillus*). There have been several reviews on bilberry (*1*), but relatively little information gathered in one place for elderberry and chokeberry.

Elderberries have been utilized in European folk medicine as early as 400 BC when Hipprocrates referred to the elder tree as his "medicine chest". Additional early remedies with elderberries include laxative (*2*), diaphoretic and oral anti-inflammatory (*3*), stomachic activity (*4*), diuretic, sudorific and antirheumatic (*5*), and treatment of bacillary dysentery (*6*). On the otherhand, Chokeberry is native to the Northeastern United States and used by both early settlers and Native Americans for much of the same. Bilberry anthocyanins have had an anecdotal history for improving night vision as well as aiding in scurvy, urinary complaints and diarrhea (*1*).

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Chemical Composition

While each of the three berries possesses a unique chemical composition, they are all high in anthocyanins, polyphenols and flavonoids. Both elderberry and chokeberry contain anthocyanins with the cyanidin aglycone, but with varying sugar substitutions. All three berries contain non-acylated anthocyanins, no organic acids are attached to the sugar moiety (*7-9*). Elderberry contains cyanidin-3-glucoside, cyanidin-3-sambubioside, cyanidin-3,5-diglucoside, and cyanidin-3-sambubioside-5-glucoside (*9,10*) while chokeberry contains cyanidin-3-galactoside, cyanidin-3-glucoside, cyanidin-3-arabinoside, and cyanidin-3-xyloside (*11,12*). Bilberry is reported to contain 15 anthocyanins comprised of five of the six anthocyanin aglycones found in nature (cyanidin, peonidin, delphinidin, petunidin, and malvidin) each with a 3-glucoside, 3-galactoside and 3-arabinoside sugar substitution (*8,13*). The berries contain differing concentrations of anthocyanins; elderberries, 664-1816 mg anthocyanins/100 g fresh weight (*14*), chokeberry, 500-1050 mg anthocyanins/100 g, and bilberry, 300–700 mg/100 g (*8*).

Elderberries, chokeberries, and bilberries contain differing flavonoid patterns as well, aside from the anthocyanins. All three contain the flavanols catechin and epicatechin, proanthocyanidins based on these subunits and phenolic acids. Elderberry also contains the flavonol quercetin-3-rutinoside (known as rutin) (*9*). Chokeberries contain quercetin (known as hyperoside) (*15*). Bilberries contain the flavonols kaemferol-3-glucoside, quercetin-3-glucoside (known as isoquercetrin), quercetin-3-rhamnoside (known as quercitrin), quercetin-3-galactoside and myricetin (*9,15*).

Anthocyanins exist in the berries as glycosides, which were long considered non-absorbable into the blood stream. In 1999, Cao and Prior (*16*) and Miyazawa *et al.* (*17*) both reported direct evidence of the absorption of the glycosidic form of elderberry anthocyanins into the human blood stream. Cao and Prior (*16*) found an anthocyanin concentration of at least 100 µg/L in the plasma, 30 min after the consumption of 1.5 g of elderberry anthocyanins and Miyazawa *et al.* (*17*) found 13 µg/L in plasma, 60 min after ingesting 2.95 mg/kg body weight of elderberry and black currant anthocyanins. Additional studies on elderberry anthocyanins have been conducted by Murkovic *et al.* (*18*), and Cao *et al.* (*19*) confirming these results.

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Oxidative and Analgesic Properties

Red berries have been shown to be strong antioxidants (*20-24*). Pool-Zobel *et al.* (*25*) reported that both elderberry anthocyanins and anthocyanidins are potent extracellular antioxidants, which may protect cells against external oxidative agents. They found that the anthocyanins are equally or more effective in this activity than the aglycones.

Red berry anthocyanins have been reported as analgesic or anti-inflammatory agents. Wang *et al.* (*26*) showed cyanidin to inhibit both Cox-1 and Cox-2 *in vivo*. Although this work was done with tart cherries, cyanidin is also the main anthocyanin aglycone found in elderberries and chokeberries. Borrissova *et al.* (*27*) reported chokeberry anthocyanins exhibited anti-inflammatory activity against histamine- or serotonin-induced inflammation in rats and Rechkemmer and Pool-Zobel (*25*) reported that chokeberry anthocyanins were able to inhibit insulin-mediated stimulation of metabolism.

Cardiovascular Activities

Oxidative modifications of low-density lipoproteins (LDL) are now recognized as one of the major processes in atherogenesis, formations of plaques in the aorta and lesser arteries. Polyphenolics from red berries are among the naturally occurring plant constituents that appear to possess antioxidant activity which act as cardiovascular protection agents (*22,28,29*). One of the principal causes of plaque formation is the aggregation of oxidized lipoproteins by macrophages, forming foam cells that adhere to arterial walls that induce plaque formation. Mechanisms that would address this problem include the prevention of lipoprotein oxidation and prevention of adhesion of foam cells to arterial walls. Abuja and collaborators (*22*) reported that anthocyanins from standardized elderberry extract were found to give *in vitro* antioxidant protection, both from copper-induced LDL oxidation and from the attaché of peroxyl radicals. Laplaud *et al.* (*29*) also reported a bilberry extract also exerted potent protective action on LDL's during *in vitro* copper-mediated oxidation.

This was shown by prolongation of the lag-phase of conjugated diene production (an indicator of the oxidation of low-density lipoproteins), reduction in lipoperoxides and thiobarbituric acid-reactive substance formation and inhibition of modification in the net negative charge of LDL. In a hamster model Bertuglia *et al.* (*28*) reported bilberry anthocyanins improved the prevention of adhesion of leukocytes to arterial walls as well as a number of other measures of blood vessel function following induced ischemia and reperfusion.

In disease states characterized in part by vascular pathologies (such as atherosclerosis, hypertension and diabetes) endothelium-dependent vasorelaxation in response to different vasodilator agonists is reduced. Vascular endothelium, which lies at the interface between the circulating blood cells and the vascular smooth muscle cells, plays a crucial role in blood flow regulation and vascular tone. Youdim *et al.* (*30*) reported that *in vitro* incorporation of standardized elderberry anthocyanins by endothelial cells significantly enhanced their resistance to the damaging effects of oxidative insult. Andriambeloson *et al.* (*31*) investigated the effects of a red wine polyphenolic extract (high in anthocyanins and polyphenols) on rat aortic rings and found that the extract stimulated the vascular endothelial generation of nitric oxide (NO). A mechanism indicated for this effect is a decrease in the endothelial release of nitric oxide (NO). In addition to red wine extract, they investigated the activities of specific anthocyanins and found that those based on delphinidin (found in bilberry), but not malvidin or cyanidin, elicited endothelial-dependent relaxation. They also noted that some flavonoids, including quercetin, cause endothelium-independent vasorelaxation, perhaps by a direct action on smooth muscle cells. Work previously reported by Bettini *et al.* (cited in (*1*)) also established that bilberry anthocyanins relaxed vascular smooth muscle. Anthocyanin containing extracts reduced the response to specific contractual agents such as serotonin and acetylcholine. Possible mechanisms put forward for this effect included stimulation of prostacyclin release in vessel walls, stimulation of endothelium-derived relaxing factor and inhibition of catechol-O-methyltransferase.

A number of mechanisms may be involved in the effects of anthocyanins on blood vessels which have relevance in such indications as atherosclerosis and other cardiovascular indications, as well as diabetes. According to Morazzoni and Bombardelli (*1*) the pharmacological results obtained for bilberry anthocyanins justified clinical application for the treatment of blood vessel fragility and altered permeability that occurred with the disease states of hypertension, diabetes and arteriosclerosis. Bilberry extracts have produced positive outcomes in a number of indications; such as improvement of ulcerative dermatitis caused by impaired circulation due to blood clots or varicose veins; reduction in edema in subjects with varicose leg veins; and improvement in capillary-venular microcirculation and lymph drainage. The authors concluded that these results validate pharmacological therapy with anthocyanins for

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varicose syndrome. Colantuoni *et al.* (*32*) examined bilberry anthocyanins in a hamster model and reported that they were effective in promoting and enhancing arteriolar rhythmic diameter changes, which plays a role in the redistribution of microvascular blood flow and interstitial fluid formation. Leitti *et al.* (*33*) reported bilberry anthocyanins had significant vasoprotective and antioedema properties in rabbits and rats. Detre *et al.* (*34*) found that bilberry anthocyanins increased vascular permeability in the skin and aorta wall more than in brain vessels. They proposed that interaction of collagen in the blood vessel wall, which plays as important role in the control of vascular permeability, with the bilberry anthocyanins may be partly responsible for the protection against the permeability-increasing action of hypertension. Bertuglia *et al.* (*28*) reported oral administration of delphinidin (present in bilberry) prevented microangiopathy in experimental diabetes. Alterations in the capillary filtration of macromolecules have been well documented in diabetic patients and experimental diabetes. Cohen-Boulakia *et al.* (*35*) reported that bilberry extract was effective in preventing the increase in capillary filtration of albumin and the failure of lymphatic uptake of interstitial albumin in diabetic rats.

Human studies have shown that elderberry extract aids in maintaining the triacylglycerol levels in conjunction with a high fat meal. Murkovic *et al.* (*36*) found that consumption of elderberry extract with a high fat meal caused no significant increases in postprandial triacylglycerols, whereas consumption of a high fat meal without elderberry extract showed a significant increase in triacylglycerols.

In a clinical trial with 105 pregnant women with complications from intrauterine growth retardation (IUGR), Pawlowicz (*37*) examined the influence of chokeberry anthocyanins on the generation of autoantibodies to oxidized low-density lipoproteins. He examined the level of oxidative stress measured by serum concentration of autoantibodies to oxidized low density lipoproteins and concluded that chokeberry anthocyanins were beneficial in controlling oxidative stress during pregnancies complicated by IUGR.

Elderberry has been reported to limit some physiological effects of stress. With human subjects, Leitner *et al.* (*38*) reported that daily consumption of elderberry concentrates reduced blood glucose levels induced by stress; increased metabolism, which may help to reduce catecholamine levels, and increased cellular oxygen consumption, shown by a lowering of pO_2 in spite of faster breathing and high pH; and a drastic decrease in ionized magnesium.

Antiulcer and Antiviral Activities

Elderberry juice and purified cyanidin-chloride have been shown to have antiulcer activity. In an *in vivo* mouse model, elderberry juice and elderberry

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extract were found to have an effective scavenger effect in relation to free-radicals inducing organ lipid peroxidation and the gastric damage induced by HCL/ethanol administration (*39*). Magistretti *et al.* (*40*) examined the antiulcer effects of purified cyanidin-chloride in rat models (cyanidin-based anthocyanins are present in bilberries, elderberries, and chokeberries). They reported that given orally, cyanidin chloride antagonized gastric ulcerations induced by acetic acid, stress, nonsteroidal anti-inflammatory drugs, ethanol, reserpine, histamine and duodenal ulceration inducted by mercaptamine. They suggested that this effect might be due to potentiating the defensive barriers of the gastrointestinal mucosa. In a rat model, administration of chokeberry anthocyanins, prior to the intraperitoneal injections of PAF and ceruleine, were reported to have a beneficial effect on the development of acute experimental pancreatitis (*41*).

Elderberry contains flavonoids with known antiviral mechanisms, including quercetin, isoquercetin, rutin, and hespiridin (*42,43*). These constituents can be found in the berries, flowers, and bark of the elder. Wacker and Eilmes (*43*) reported that incubation of cells with flavonoids for 6-8 h prior to addition of influenza virus protected healthy cells. Anti-viral activity of elderberry has been documented by Zakay-Rones and colleagues (*42*). Their study had two components: *in vitro* measurement of the cytopathic effect of the extract on virus replication and agglutination of red blood cells by the extract, and *in vivo*, a clinical trial of influenza patients. The elderberry extract was shown to be effective *in vitro* against 10 strains of influenza virus and the clinical trial showed a 3-4 day reduction in flu symptoms due to the elderberry extract. Vivian *et al.* (*44*) studied the effect of elderberry extract on cytokine production. They concluded that it activated the healthy immune system by increasing inflammatory cytokine production and proposed that it might be beneficial to the immune system activation and in the inflammatory process in healthy individuals or in patients with various diseases.

Vision Related Activities

Morazzoni and Bombardelli (*1*) compiled clinical studies on the therapeutic uses of bilberry relative to vision. Since various forms of bilberry have been available in Europe for several decades, a vast number of studies dedicated to the use of bilberry in vision related problems have been published there starting in mid-1960. Some of the vision related investigations with bilberry fruit preparations have been on patients with pigmentary retinitis (*45*) and diabetic retinopathy (*46,47*), hemeralopia (diminished vision in bright light) (*48*), macular degeneration, diabetic retinopathy, retinal inflammation, or retinitis pigmentosa (*49*), progressive myopia (*50*), various retinopathies (*51*), myopia, glaucoma, or retinitis pigmentosa (*52*), diabetic and/or hypertensive retinopathy

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(*53*), and polyneuritis due to vascular insufficiency (*54*); all of which showed improvement when taken in combination with beta-carotene. Possible mechanisms of action for bilberry's effects on ophthalmic conditions include its ability to protect against the breakdown of rhodopsin (retinal purple), a light sensitive pigment located in the rods of the retina, and its ability to regenerate rhodopsin (*55*). This makes bilberry a possible aid for those with poor night vision (*56*) or in dim light (*57*). It may also provide vasoprotection by decreasing capillary fragility and permeability (*58,59*). In contrast, researchers have also published results refuting the effect of bilberry supplementation on night vision acuity and contrast sensitivity (*60*).

Aside from bilberry's legendary role in improving night vision, it has been used to help in the treatment of glaucoma, cataracts, and retinopathy (*61-63*). Bilberry anthocyanins can act as antioxidants in the retina of the eye. Retinal protection may be aided by anthocyanidinic retinal phosphoglucomutase and glucose-6-phosphatase inhibition (*64*). Bilberry has also been shown to strengthen capillaries and reduce hemorrhaging in the retina (*65*). The anthocyanins may protect both the lens and the retina from oxidative damage.

Anti-cancer Activities

Another avenue of research is the use of red berry flavonoids or polyphenolics in anti-cancer activity. Christidou *et al.* (*66*) examined standardized chokeberry extracts on malignant human brain tumor cell lines *in vitro* and found increased cell death and downregulation of MMP-9 secretion. Increased cell death with chokeberry as well as bilberry extracts was also noted. They proposed that chokeberry flavonoids might be promising candidates as novel treatments for brain tumor therapy. Bomser *et al.* (*67*) reported that a hexane/chloroform extract of bilberry exhibited potential anticarcinogenic activity as evaluated by *in vitro* screening tests. Leonardi (*68*) utilized bilberry extract in the therapeutic treatment of fibrocystic mastopathy and reported that therapy for three months in patients with mastodynia, consequent to fibrous mastopathy, was efficacious in reducing symptoms and mammary tension.

Red berry products can be found in a variety of forms including: juices, concentrates, powders, fibers, and extracts. These products are all processed differently and thus contain varying amounts of bioactive ingredients. The studies described above used varying forms of the berries. Many studies describe the anthocyanins as the causative agent responsible for a given activity, but these fruits are a complex mixture of flavonoids and other compounds. Thus it is difficult, at best, to ascertain if the anthocyanins are solely responsible for the activity or if the anthocyanins are working synergistically with other flavonoids to cause the activity. Therefore it is important for researchers to fully

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describe the sample they are experimenting with and include any compositional data.

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Chapter 11

Stability of Juice Beverages as Affected by Pectinmethylesterase

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Orange juice concentrates are often used as a base in juice blends with other fruit juice beverages as well as milk drinks. Enrichment of juices and other beverages with minerals in addition to the use of concentrates from citrus, kiwi, mango, passion fruit and other fruits provide a ready source of tailored juice beverages that are high in phytochemicals; however, mixed juices or juice blends are often not stable upon blending or storage. Pectinmethylesterase (PME) is responsible for blockwise de-esterification of pectin and is implicated in clarification of citrus juices and juice-containing beverages by initiating the formation of calcium reactive pectic acid. Since cloud instability or clarification is a major defect affecting the appearance of juices and concentrates, identification of the causative factors relevant to the juice and nutritional beverage industry is appropriate.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Pectinmethylesterase (PME) occurs naturally in all plant tissues. In citrus, disruption of the cell wall matrix releases PME, enhances the de-esterification of pectin, the formation of insoluble, large molecular weight calcium pectate aggregates, and "cloud" loss (clarification) (*1,2*). Several forms of PME have been separated and characterized from lemons (*3*) and navel oranges (*4*). Other thermolabile (TL-PME) and thermostable (TS-PME) PME isozymes have been identified (*5*) that differ in low pH tolerance, hydrophobicity, secondary structure, clarification rates (*6-10*) and gelling characteristics of pectin (*3*). Different physico-chemical properties of PME are implicated in varied rates of clarification. In spite of the ostensible role of PME in quality of juices, no direct correlation between PME, degree of de-esterification (%DE) of pectin, and clarification of juices has been reported.

Citrus juice is a colloidal dispersion of sugars, cellular organelles, membranes, chromatophores, oils, flavonoids, and cell wall fragments; however, citrus cloud has not been described in colloidal terms. Citrus juice cloud is typically described by compositional and particle size analysis. After commercial juice extraction and finishing, large particulate material composed of juice sacs sediment and this substance is termed settling pulp. The material that remains suspended is the citrus juice cloud, which has a particle size of less than 2 μm (*11*). Cloud analysis yields an approximate composition of 52% protein, 4.5% pectin, 25% lipid, 2% hemicellulose, and 1.5% cellulose (*12-15*).

PME modification of pectin plays a major role in cloud stability of not only citrus juices, but also juice containing beverages, fortified sports drinks, and acidified milk drinks. Further, it offers the potential to prepare tailored pectins of specific total charge and unique charge distribution properties. The functional performance of pectins as a stabilizer of colloidal dispersions can be better predicted and controlled with more complete information on the impact of PME activity. To prepare protein fortified drinks such as acidified milk drinks, sports drinks, and juice beverages, stabilizers like pectin should prevent sedimentation and serum separation, imparting a smooth, creamy mouthfeel. In addition, the stabilizer should moderate particle size of dispersions and tolerate process treatments, such as pasteurization. In general, pectins that are good stabilizers of acidified drinks have a high molecular weight and low net charge. High ester pectins that are calcium sensitive and anchor pectin to the protein surface have the best stabilizing properties and may be obtained by chromatographic separation of desirable pectins (*16*) or by enzymatic modification of native pectins (*17*). Modified pectins likely have different physical properties depending on the extent of modification and the length of de-esterified blocks. The distribution of esterification plays an important role in pectin interactions with cations.

In this chapter, information and research that indicates PME activity is influenced by extrinsic factors, including cations, non-PME protein and PME-

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pectin complexes are provided. Clarification of citrus juices and juice containing beverages, as well as the instability of pectin stabilized drinks is attributed to pectin interactions. Factors that influence PME activity, the role of PME in modification of pectin charge and stability of dispersions, are emphasized.

Materials and Methods

Source of Material

PME was extracted from citrus (Valencia orange or Marsh grapefruit (MGF)) using 0.25M Tris-Cl-, 0.1M NaCl, pH 8.0 (1:4) (*18*) or 1 M NaCl without pH adjustment (*19*). The PME extracts were chromatographed on Hi-Trap SP, Hi-Trap Heparin Sepharose, and ConA (Amersham-Pharmacia Biotech, Piscataway, N.J.) according to established procedures (*20*). The alkaline extraction procedure extracts TL-PME and TS-PME in Valencia and MGF. The endogenous pH extraction procedure is selective for TS-PME from Marsh grapefruit pulp but is not selective for TS-PME extracted from Valencia (Wicker, unpublished results). Protein content was estimated by the dye binding method (*21*). PME activity was determined by titration at pH 7.5 in 1% pectin, 0.1M NaCl at 30°C and was expressed as microequivalents of ester hydrolyzed per min.

Modified pectins and interaction with milk proteins

A high methoxyl, calcium reactive pectin was obtained from Copenhagen Pectin A/S (Copenhagen, Denmark). It had 92% uronic acid, estimated MW of 244,700, and 73% degree of esterification (DE). Sodium caseinate was prepared by acid precipitation from Nilac skim milk powder (NIZO, Kernhemseweg, the Netherlands) and high β-lactoglobulin; whey protein isolate was obtained from Amor Proteins (St. Brice enCogles, France). The protein dispersions were acidified to pH 4.0 with glucono-delta-lactone and homogenized with pectin to a final concentration of 0.1, 0.25 or 0.35% pectin. The supernatant was collected after separation in a microfuge centrifuge and analyzed for MW with a Viscotek 250 Triple Detection (RI, viscosity, and light scattering (Oss, Netherlands) with Biogel TSK 60-40-30XL columns in series, using 0.2M citrate, pH 4.0.

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Results and Discussion

The lack of correlation between PME activity and clarification presents ambiguity in the PME model for juice clarification. When clarification occurred in pasteurized juices, it was attributed to a variety of factors, including *a priori* PME activity, residual TS-PME activity, improper inactivation of PME in pulp of pulp added juices, and PME isozyme activity. The amount of PME or TS-PME detectable in juice was not well correlated with quality indicators of juice such as percentage of settling pulp, Brix, pH, and titratable acidity, among others. (*22*). Versteeg *et al.* (*5*) suggested that juice clarification at 4°C is primarily due to TS-PME, thus subsequent research was directed towards quantifying, characterizing, and inactivating the TS-PME. A study by Cameron *et al.* (*23*) confirmed that heated extracts of juice, peel, and rag (100% TS-PME) induced the most rapid clarification. However, their results clearly indicated that % thermostable activity could not be used solely to estimate propensity to clarification, since tissue specific differences were observed. Recent studies that have partially purified PME and tested for juice clarification are summarized in Table I. The total units of activity (microequivalents of ester hydrolyzed per min) per mL of extract or specific activity (microequivalents of ester hydrolyzed per min per mg of protein) are reported if provided, and the time of change in transmittance was estimated from the data presented. Cameron *et al.* (*23*) presented strong evidence that insoluble PME extracted from juices, which was only 5.6% thermostable, clarified juice at equal rates to soluble PME that was extracted from pulp and heated (100% thermostable). They also observed that the source (juice, rag, or peel) and solubility of PME influenced PME induced clarification. At 4°C, insoluble PME extracted from juice (DP) or soluble PME from peel (DS) clarified juice in the shortest period of time. In a later study, Cameron *et al.* (*24*) showed that less than 1.5U PME per mL of fresh juice resulted in a decrease in absorbance within a week and related it to peel residue in the juice of "hard extracted" juice. Regardless of extraction method, PME activity estimated at juice pH (4.5) was a better predictor of clarification than PME activity estimated near the pH optimum (7.5). This suggests that the mechanism of de-esterification in juice may be different as reported for the mechanism of de-esterification of commercial pectins by apple PME at pH 4.5 and 7.5 (*25*).

Some of the reported PME isozymes are likely to be PME-pectin complexes (*3,26*) that influence isoelectric focusing and ion exchange separation of PMEs, detectable activity, thermostability, and rates of clarification. PME can be solubilized from an inactive pectin complex (*27,28*) and activate apparent PME activity. At higher concentrations, cations inhibit PME by competitive displacement. In addition, the effect on PME activity is cation specific (*29-32*). Calcium chloride has a broad concentration effect on activity (Fig 1A) unlike

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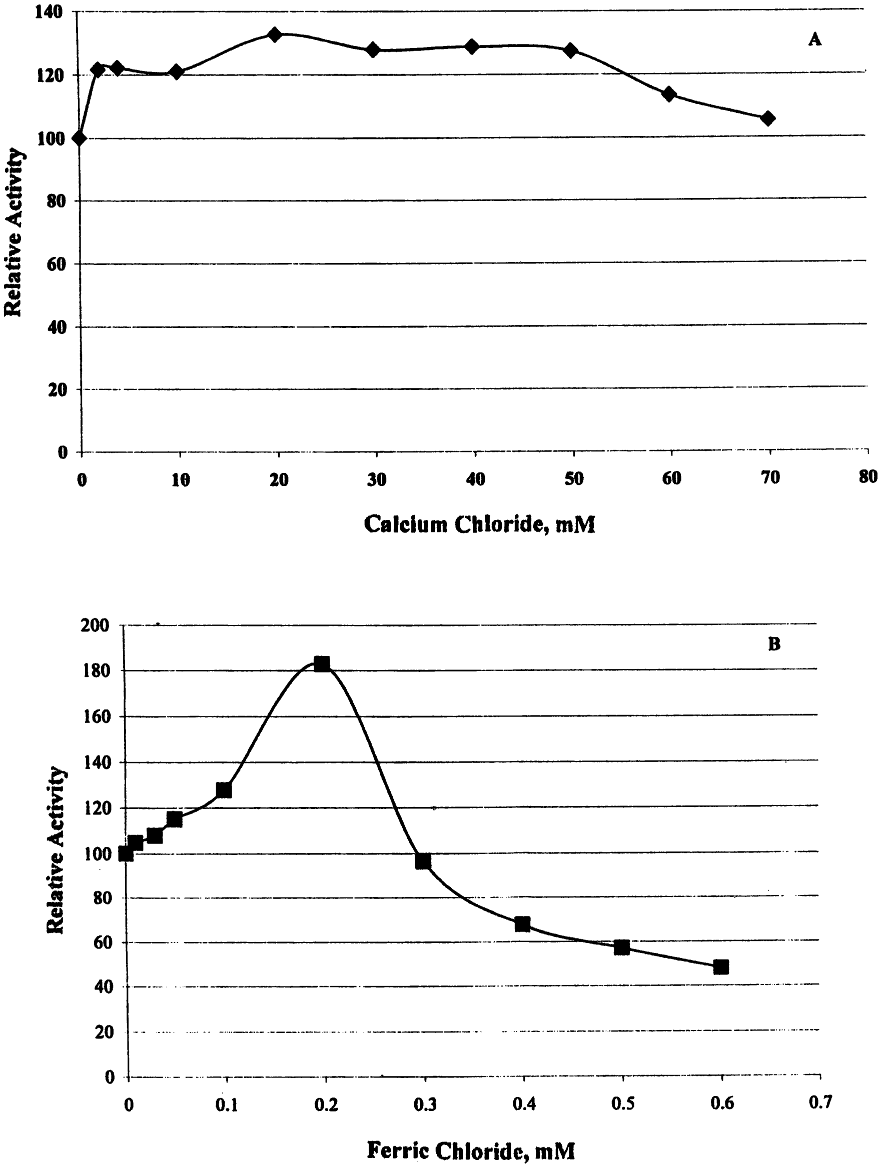

Figure 1. Effect of calcium chloride and ferric chloride on PME activation and inhibition. Adapted from Leiting and Wicker (1997).

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Table I. Source, total activity, specific activity and onset of clarification reported for citrus juices, stored at 4°C

Source	*Activity*	*Specific activity*	*Clarification*[a]	*Reference*
Valencia orange[a]	U/μL[b]	U/μg pro[b]	5U/mL juice OD < 1.25	Cameron *et al.* 1997
Peel DS	1.47	0.11	yes, 21d	
Peel DP	0.26	0.11	no	
Rag DS	10.84	0.89	no	
Rag DP	0.55	0.23	no	
Juice DS	1.64	0.04	no	
Juice DP	0.30	0.25	yes, 14 d	
Fresh Valencia juice[c]	Units/ml		OD < 1.25	Cameron *et al.* 1999
Soft	1.1	NR	no	
Medium	1.4	NR	yes, 6 d	
Hard	1.2	NR	yes, 6 d	
Valencia juice[d]	Units/ml			Cameron *et al.* 1999
PME-S - pH 7.5	49.1	NR	yes, 12d, 1U/mL	
PME-S - pH 4.5	(23.9)	NR	no, 0.5U/mL	
PME-M – pH 7.5	89.7	NR	no, 1U/mL	
PME M – pH 4.5	(27.9)	NR	yes, 15 d, 0.5U/mL	
PME-H – pH 7.5	91.9	NR	no, 1U/mL	
PME –H- pH 4.5	(26.9)	NR	yes, 10d, 0.5U/mL	
Valencia pulp[e]	Units/ml	U/mg Pro	2U/mL juice	Ackerley *et al.* 2002
BP++	281	311	no	
BP+	290	323	no	
UBP-	1557	483	yes, 7d	
CE-F	3771	667	yes, 11d	
Valencia pulp[f]	Units/ml	U/mg Pro	1.2U/mL juice	Wicker *et al.* 2002
PME	290	323	no	
PME, 4.2 mM Ca^{++}	290	323	yes, 2 d	
PME, 16.7 mM Ca^{++}	290	323	yes, 1 d	
PME, 16.7 mM Sr^{++}	290	323	yes, 1 d	

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PME, 10 mM spermidine	290	323	yes, 2 d	
Valencia orange pulp[g]		U/mg Pro	2U/mL of juice	Wicker *et al.* 2000
Frac 1	NR	900	no	
Frac 2	NR	1600	no	
Marsh grapefruit pulp[g]		U/mg Pro	2U/mL juice	Wicker *et al.*
Frac 1	NR	40	yes, 6 d	2000
Frac 2	NR	300	yes, 15	

[a] Because of differences in laboratory protocols, onset of change in absorbance and transmittance was arbitrarily set at 1.2 or 30%, respectively. [b] The PME extract was derived from orange peel, rag, or juice and DS or DP refers to the PME that remained soluble or precipitated after dialysis, respectively. [c] The activity was reported in U/μL, not U/mL in this reference. Specific activity was expressed per μg protein. [d] The terms soft (S), medium (M), and hard (H) refer to extraction and finishing pressure during processing. PME activity was measured at pH 7.5 or pH 4.5. [e] BP++, BP+, and UBP- refer to fractions with decreasing affinity for cationic exchanger (HiTrap SP), respectively. CE-F refers to the unfractionated, crude PME extract. [f] PME that was partially purified by HiTrap SP was added to juice with or without cations at concentrations as indicated. [g] PME was partially purified using several column fractionation techniques to achieve different specific activities and added to juice.

ferric chloride (Fig 1B), which has a narrow range of concentration for activation of PME. Sun and Wicker (*31*) proposed that an isokinetic temperature near the assay temperature for calcium activated reactions was responsible for the broad calcium concentration effect. In addition, cations uniquely influenced the free energy of the PME-pectin complex (*32*). Separation of PME from pectin complexes was not easily accomplished (*33*) and pectin co-eluted with PME during purification (*19*). The confounding role of PME-pectin complexes on juice clarification was further demonstrated in a subsequent study. Presumptively, since cations influence activity, cations will also influence the rate of clarification of juices. Pasteurized juices with 1.2 U of Valencia TL-PME per mL of juice were cloud stable for up to 15 days. However, the addition of cations, such as calcium, strontium, or spermidine, initiated clarification within 2 days (Table I), presumably by release from an inactive enzyme-substrate complex (*34*). Interestingly, some purification schemes result in an increase in TS-PME activity (Table II). When MGF TS-PME was separated on Hi-Trap SP with an initial NaCl concentration of 0.1M, 0.25M or 0.5M, the %TS PME ranged from less than 100% to 167% in fractions of TS-PME (*19*). The authors proposed that ion exchange separated a component, such as pectin, an inorganic cation, or protein, that influenced the amount of TS-PME activity in the later eluting fractions.

The role of non-PME proteins on clarification has not been well studied considering the role proteins play in apple juice cloud and the amount of protein in citrus cloud. Although the role of protein in citrus cloud is probably different from that of apple cloud (*11,14*), protein induced clarification has been proposed for peel (*35,36*) as well as for juice (*37,38*) extracts. This is primarily because pectinase treated juices are more prone to coagulation, and ultrastructural analysis show the formation of protein coagulates. Other studies have related lower purity of PME extracts with greater clarification of juices. With partially purified Marsh grapefruit PME, clarification was observed 9 days earlier with an extract that had almost 10 fold lower (40U/mg protein) specific activity, ie. more non-PME protein, than observed with a PME of greater purity (300 U/mg protein) and Valencia PMEs with very high purities and specific activities (> 900 U/mg protein) did not clarify juice (*39*). The affinity of cationic Valencia TL-PME to HiTrap SP was inversely related to clarification (*40*). TL-PME that bound SP on the first or second pass showed no evidence of clarification after 15 days when added to pasteurized juice at 2 U per mL of juice. TL-PME that did not bind SP showed evidence of clarification after 7 days. The crude extract initiated clarification after 11 days (Table I). The authors reported that the PME extracts that clarified juice contained a 27 kD protein band that was absent in fractions that did not clarify juices.

The nature of PME sensitization of pectins and clarification of juices was evaluated by measuring the total charge as %DE required for pectin calcium

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Table II. Change in thermostable PME activity after fractionation on HiTrap SP with increasing amounts of NaCl in the start buffer

Fraction No.	*Specific Activity (U/mg Protein)*	*% TS-PME*
	0.1M NaCl	
Extract	56	80
6	170	0
8	203	76
11	463	111
12	387	87
	0.25M NaCl	
Extract	56	80
8	85	94
11	243	101
12	162	78
	0.5M NaCl	
Extract	17	109
7	60	93
8	53	158
11	535	167

Adapted from Corredig *et al.* 2000. Initially, the crude TS-PME extract contained 80 to 109% thermostable PME activity. After fractionation, the percentage of %TS-PME increased in later eluting fractions.

sensitivity and juice clarification. Plant PME results in "blockwise" and alkali saponification or fungal PME results in random de-esterification patterns (*41*). The blockwise nature of PME de-esterification of commercial pectins is credited with calcium sensitivity of pectin and sensitizing juices to clarification. PME or alkali de-esterified pectins influenced the rate of clarification of juices with pectins of differing %DE values, and pectins varied in the ability to cause clarification depending on pH (*42*). Pectins at 42% DE de-esterified by PME or alkali did not clarify Valencia juice. However, even in the absence of clarification, sensitized pectins increased particle size of juices, and PME sensitized pectins increased particle size to a greater extent than alkali sensitized pectins (*43*). The reported %DE of pectins from clarified juices varies widely; Krop (*44*) noted that an average %DE values of about 20% for clarified juice pectins. A study by Ackerley (*40*) reported that pectins extracted from stable juices had a 19%DE while pectins from clarified juices had a %DE close to 13%. Because the intramolecular de-esterification of a small number of pectin molecules may sensitize juices to clarification, no single value of %DE can accurately characterize pectins of clarified juice (*44*).

In addition to the effect on total charge, distribution of charge is influenced by the method of de-esterification. Alkali saponification or fungal PME de-esterification results in a random de-esterification pattern, whereas plant PME results in a "blockwise" distribution of de-esterified regions (*41,45*). Pectins with nearly identical total % DE elute from ion exchange chromatography differently depending on the method of de-esterification (*46*). Pectin treated with citrus PME was broad and not proportional to total %DE. In addition to the type of PME, pH also influences the degree and type of de-esterification that is observed. Pectins modified by fungal PME, pH 4.5, apple PME, pH 4.5 or apple PME at pH 7.0 have unique patterns of de-esterification. Fungal PME, pH 4.5 or apple PME, pH 4.5 de-esterifies in multiple chain attack, but in a random or sequential manner, respectively. Apple PME at pH 7.0 has a single chain attack in a sequential manner (*25*).

The total charge and charge distribution of pectins influences interaction with proteins in fortified drinks, such as acidified milks, drinkable yogurts, fruit flavored whey or other dairy beverages. At concentrations greater than a critical pectin level, dispersions are stable, not prone to sedimentation or serum separation. However, overdosing of pectin leads to high viscosity, and may have an unacceptable, "slimy," mouthfeel (*47*). High methoxyl pectin stabilizes acidified caseinate dispersions more effectively than low methoxyl pectin (*48*). The stabilizing effect of pectin on acid milk drinks may be due to several factors. Pereyra (*48*) proposed that the methoxylated galacturonic acid units do not interact with the casein, extend outward from the casein particles, and stabilize by solvent interaction. Barnes (*49*) showed that the particle size of stable dispersions were small (< 2 μm) and monodisperse.

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Most likely, the size of pectin-protein aggregates plays a major role in separation of unstable dispersions. When calcium sensitive pectin was used as a stabilizer of acidified whey or caseinate dispersions, large aggregates were formed. Most likely, aggregates of pectin-caseinates were larger and less stable than pectin-whey aggregates. Elution profiles from gel permeation chromatographic (GPC) separation of caseinate-pectin dispersions were characterized by a poorly resolved, polydisperse aggregate (Figure 2A). In these samples, virtually all of the casein precipitated in the pellet after centrifugation at 4000xg in a microfuge tube, presumably due to larger aggregate size. In contrast, a greater proportion of whey-pectin dispersions remained suspended after microfuge centrifugation, and intermediate aggregates were observed in the light scattering signal (Figure 2B). As many as two to four intermediate MW species were observed in pectin-whey dispersions, but the occurrence and the MW was variable and ranged from about 50,000 to over 2 million daltons, depending on pectin concentration and other factors. The MW of pectin alone was estimated to be 244,700 Da (data not shown). Interaction of a high methoxy, calcium sensitive pectin with caseinate or whey at acidified pH formed large, variable aggregates. These results suggest that pectins interact with caseins and whey by different mechanisms, and that casein-pectin interaction form larger, more unstable aggregates that precipitated prior to MW analysis.

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Conclusions

The PME and calcium pectate model for citrus juice clarification warrants expansion. While it is conceivable that clarification may eventually be explained based on factors intrinsic to different PME isozymes and mechanisms of action, evidence has accumulated that extrinsic factors profoundly influence the mechanism of action of PME isozymes. The role of these extrinsic factors have not been fully elucidated but include organic and inorganic cations, non-PME protein, pH and temperature of de-esterification, and method of processing. Likewise, these extrinsic factors will influence the properties of pectins used as stabilizers in functional beverages, such as pectin stabilized milk drinks. The mechanism of de-esterification can be used to produce tailored pectins with specific and controlled stabilizing properties.

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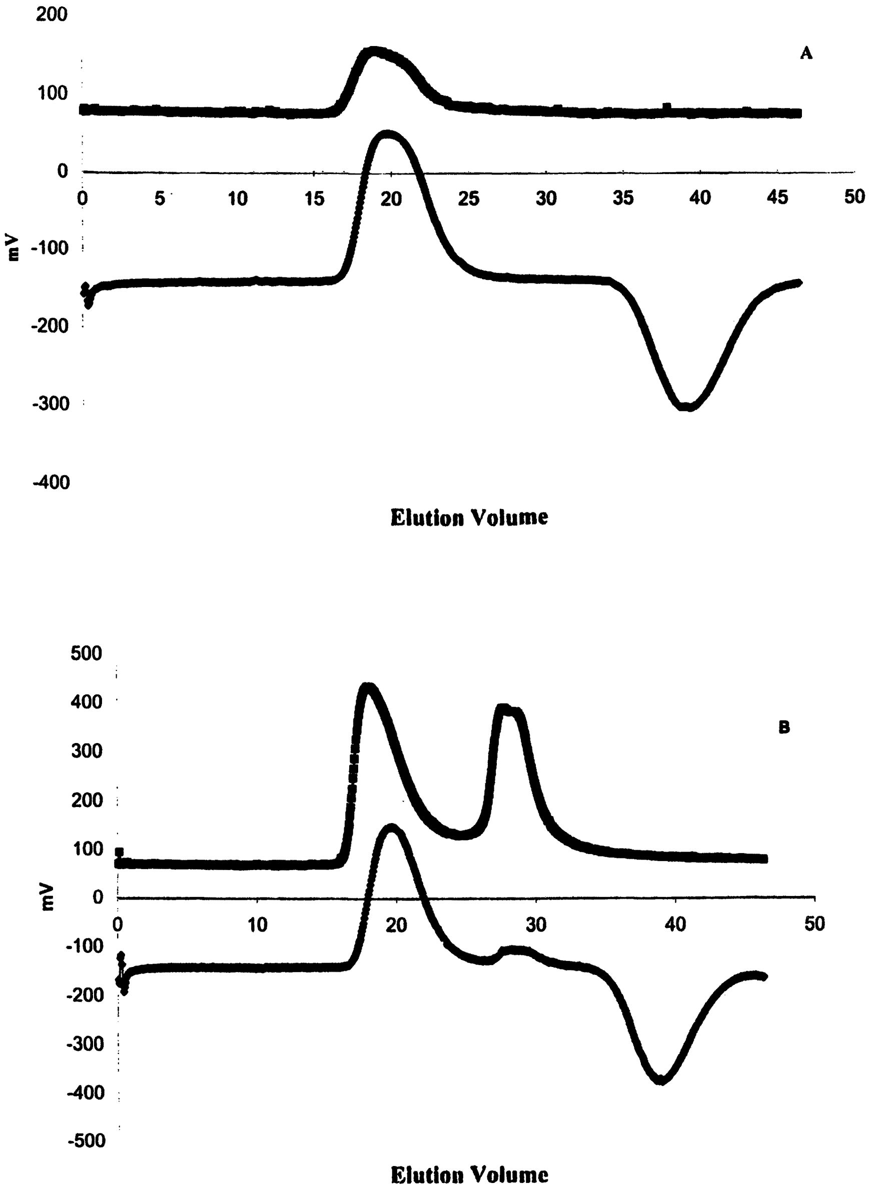

Figure 2. Elution of pectin-protein aggregates that remained in suspension after microfuge centrifugation and separated by size exclusion chromatography. The upper curve denotes the light scattering and the ower curve denotes the viscosity signals. (A) casein-pectin (B) whey-pectin (C) pectin only.

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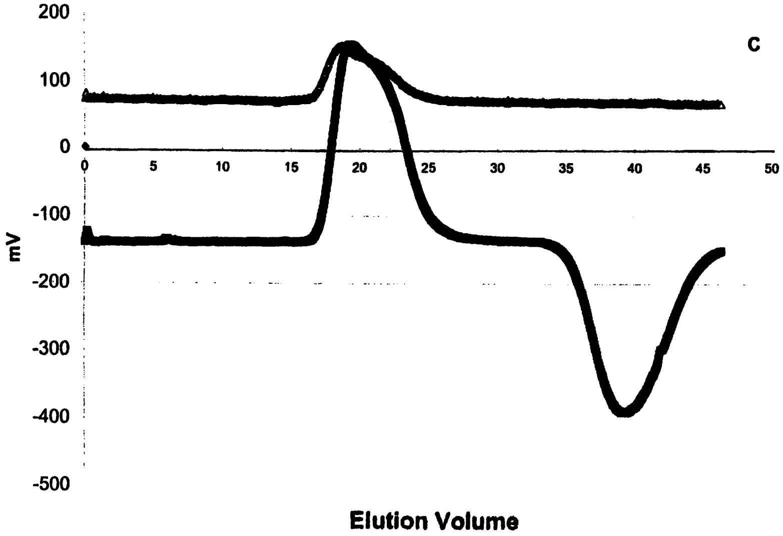

Figure 2. *Continued.*

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Chapter 12

Antioxidant Activity of Blueberry and Other *vaccinium* Species

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Fruits, especially berries are a rich source of phenolic and polyphenolic compounds that are responsible for their protection against oxidative stress. Recent interest in the possible protective effects of dietary antioxidants against human degenerative diseases has prompted investigation of food constituents, including blueberries. The potential health benefits of berries, particularly blueberries are discussed in the context of their antioxidant potential and with reference to blueberries from Canadian crops grown in Newfoundland, Nova Scotia and Quebec.

Introduction

Epidemiological studies have shown that consumption of fruits and vegetables as well as their juices may reduce the risk of several forms of cancer as well as cardiovascular diseases (*1*). In this relation, phytonutrients present in fruits and vegetables have been found to possess health promoting properties. The mode of action of such components may involve prevention of oxidative damage to biomolecules and hence degenerative diseases of aging. The compounds that are responsible for rendering such effects possess antioxidant properties and are often phenolic and polyphenolic in nature.

Phenolics and polyphenolics are the largest group of secondary metabolites originating from phenylalanine, and to a lesser extent, in certain plants, from tyrosine (*2*). Their production is facilitated by the action of phenylalanine ammonia lyase (PAL) and tyrosine ammonia lyase (TAL), respectively, corresponding products of which are cinnamic acid and *p*-coumaric acid. This series of compounds are known as phenylpropanoids (C_6-C_3). The chalcones

In Nutraceutical Beverages; Shahidi, F., et al.;
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(C_6-C_3-C_6) are subsequently produced via condensation of phenylpropanoids with 3 molecules of malonyl coenzyme A while benzoic acid derivatives are produced via the loss of a C_2 moiety from C_6-C_3 compounds. Cyclization of chalcones leads to the formation of different subclasses of flavonoids. Further condensation of flavonoids and/or phenolic acids leads to the formations of condensed and hydrolyzable tannins.

In this contribution, special attention is paid to potential health benefits of berries, particularly blueberries, as reflected in their antioxidant potential and content of phenolics and polyphenolic compounds.

Blueberries

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Blueberries are *Vaccinium* species which grow wild in many countries around the world. However, Canada and United States supply about 95% of the fruit used by the food industry (*3*). North American farmers harvest about 47 million kilograms of blueberry annually out of which nearly one third is marketed as fresh fruit; another one third is frozen and the rest goes into bakery products (*3*). The main commercial *Vaccinium* fruits in North America include highbush blueberries (*V. corymbosum*), lowbush blueberries (*V. angustifolium*), rabbiteye blueberries (*V. ashei*), cranberries (*V. membranaceum and V. ovalifolium*) and partridgeberries (*V. vitis-idaea*). Lowbushberry is grown commercially in eastern Canada and in the northeastern United States (Maine). The annual production of this berry type ranges from 23,000 to 47,000 tons. The most important species in Europe include bilberries (*V. myrtillus*) and lingonberried (*V. vitis-idaea*), the former one being best known for its health effects. Berries are rich in flavonoids and phenolic acids. Prior *et al.* (*4*) reported that blueberries (*Vaccinium spp*) have high antioxidant capacity compared to other fruits (*4*). The reports of high antioxidant capacity of wild and other blueberries have sparked considerable popular interest among researchers and consumers (*5*). The other berries with significant antioxidant capacity include cowberry (*Empertum nigrum*), cloudberry (*Rubus chamaemorus*), whortleberry (*V. uligonosum*), aronia (*Aronia melanocapra*), cranberry (*V. oxycoccus*), rowanberry (*Sorbus aucuparia*), all being wild berries, while the cultivated berries such as red currant (*Ribes rubrum*), black current (*Ribes nigrum*), and raspberry (*Rubus idaeus*) showed a lesser activity.

Antioxidant Activity of Blueberries

Blueberries have attracted special interest to the scientists because of their high antioxidant capacity. The phytochemicals in plant tissues responsible for the antioxidant capacity can largely be attributed to the vitamin C, tocoperhols, phenolics, anthocyanins and other flavonoid compounds (*6*) which may vary greatly in their content and profile among various fruits and vegetables. Compared to other fruits and vegetables a higher antioxidant capacity has been reported for blueberries (Table I) (*4*). Lowbush wild blueberries are one of the best fruit sources of anthocyanins (*7*) and are known to exhibit one of the highest recorded antioxidant capacities of various fruits and vegetables *in vitro* (*8,9*). Wild blueberries are relatively low in antioxidant vitamins and minerals (*10*). Their *in vitro* antioxidant capacity has been attributed to the presence of high concentrations of phenolic compounds, particularly anthocyanins (*4*). Prior *et al.* (*4*) have indicated that oxygen radical absorbance capacity (ORAC) values in highbush cultivars and other *Vaccinium* species range from 13.9 to 45.9 μmol Trolox equivalents/g of fresh fruit. The wide range of values suggests that antioxidant levels in blueberries could be improved through breeding practices. Prior *et al.* (*4*) also indicated significant correlations of 0.72 and 0.92 between ORAC values and anthocyanin and phenolics, respectively.

Table I. Total phenolic, anthocyanin and ascorbate contents of blueberry and bilberry

Cultivar	*Anthocyanin (mg/100g)*	*Phenolics (mg/100g)*	*Ascorbate (mg/100g)*
	***V. corymbosum* L. (Southern Highbush)**		
Reveille	62.6 ±3.8	233±1.5	4.9±0.1
Blue Ridge	110.8±3.5	347±10.9	9.5±0.8
Cape Fear	157.3±5.2	331±10.3	NA
Pender	157.4±3.7	349±7.1	NA
Bladen	130.9±5.5	473±10.7	NA
	***V.angustifolium* (Lowbush)**		
Cumberland	103.6±0.9	295±13.2	8.0±0.2
Lowbush (PEI)	91.1±0.7	313±6.4	3.6±0.2
Blomidin	179.6±3.4	453±18.5	1.7±0.2
Fundy	191.5±2.5	433±45.5	4.3±0.1
Lowbush (NS)	175.0±1.6	495±3.5	9.7±0.1
	***V. myrtillus* L.**		
Bilberry	299.6±12.9	525±5.0	1.3±0.1

Adapted from Prior *et al.* (4)

Prior and co-workers (*4*) reported that increasing maturity of blueberries at harvest yielded high antioxidant, anthocyanin and total phenolic contents. Maturity at harvest had a marked effect on ORAC, total anthocyanins, and total

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phenolics of the berries for the cultivars of Brightwell and Tifblue of rabbiteye blueberries. Berries harvested immediately after turning blue had lower ORAC and total anthocyanins than their well matured counterparts that were harvested 49 days later. ORAC and total anthocyanins increased 224 and 261%, respectively, in the Brightwell while in Tifblue the increase was 164 and 176%, respectively. Prior *et al.* (*4*) also indicated that underripe blueberry fruits with a relatively low concentration of anthocyanin might still have a high level of ORAC value, suggesting that other phenolic components present in underripe fruits contribute substantially to ORAC. To the contrary, Wang and Lin (*11*) indicated that blackberry, Jewell black raspberry and strawberry fruits harvested during the green stage consistently yielded the highest ORAC values.

In the blueberries investigated in this work (Table II) ORAC values ranged between 9.51 and 25.96 μmol of Trolox equivalents/g fresh fruit (results not shown). This is in the range of values reported by others (*4,11*).

Kalt *et al.* (*5*) reported that pH of blueberry juice affects the content of anthocyanin, polymeric color, total phenolic content and antioxidant capacity. The high level of anthocyanins (118 μg/mL as malvidin 3-glucoside equivalents) present is consistent with the presence of the flavylium cation which is a most intensely colored compound to quinonodial pseudobase, and chalcone forms, which are pale in color. The high percentage of polymeric pigments in juices of pH > 1 indicates the greater self-association of anthocyanin molecules at higher pH values (*5*). However, it is not clear whether anthocyanin self-association affects antioxidant capacity or not (*5*). Oxygen has been identified as an important factor in destabilizing anthocyanins in processed blueberry products (*7*). Kalt *et al.* (*5*) reported that introduction of oxygen to blueberry juice had a marked effect on monomeric anthocyanins, total phenolics and antioxidant capacity. In addition to direct oxidation, anthocyanins with an ortho-diphenolic B ring can be degraded by ortho-quinones formed by the oxidation of chlorogenic acid by polyphenol oxidase (*12*).

Ehlenfeldt and Prior (*13*) evaluated 87 highbush blueberry cultivars for their antioxidant activity. The mean ORAC value was for samples examined 15.9 Trolox equivalents (TE) per gram of fresh fruit weight while ORAC/cm^2 value was 4.2 TE/cm^2. Furthermore, the ORAC/g of fresh weight correlated with total phenolics (r=0.76, 87 samples) and anthocyanins (r= 0.57, 87 samples). Mean ORAC value of blueberry leaves across 87 samples was 490 TE/g on a wet weight basis. A significantly high correlation existed between leaf ORAC and leaf phenolic values (r = 0.87, 77 samples). Leaf ORAC had a low but significant correlation with fruit phenolics and anthocyanins but not with fruit ORAC. Prior *et al.* (*4*) demonstrated that there was a great variability in ascorbate concentrations (1.3-16.4 mg/ 100g) between cultivars and species of blueberry. Furthermore, no consistent pattern was describable between ascorbate concentration and ORAC, anthocyanins or total phenolic content. The

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Table II. Total phenolic content and radical scavenging activity as measured by a luminescence technique of blueberries grown in Canada

No.	*Sample*	*Total phenolics*[1] *(mg/100g)*	*Inhibition*[2] *(%)*
1	Wild blueberries from Central Bonavista (NL)	162.0±2.3	56
2	Farmed blueberries from Avalon (NL)	278.2±1.6	75
3	Wild blueberries from Avalon (NL)	152.1±3.1	55
4	Wild blueberries from Mont Valin (Quebec)	83.6±1.2	36
5	Farmed blueberries from Nova Scotia	177.0±3.3	41
6	Wild blueberried from Central Bonavista North (NL)	151.3±1.3	46
7	Farmed blueberries from Central (NL)	155.0±.9	57
8	Farmed blueberries from Grand Falls (NL)	145.2±2.1	41

[1] Total phenolic content expressed on the weight of fresh fruit

[2] Radical scavenging capacity was measured by Photochem™ and expressed as inhibition%.

NL; Newfoundland and Labrador.

fruits with broken skins had significantly lower ascorbate concentrations due to possible oxidation upon exposure to oxygen. Using an ORAC value for ascorbate of 5.6 mmol TE/g, it was calculated that the antioxidant capacity contributed by ascorbate to the total antioxidant capacity, measured as ORAC, was 2.3% for highbush and rabbiteye blueberries. Ascorbate in lowbush berries contributed only 1.5% while in bilberry sample was only 0.2% (Table I). Thus, it is clear that ascorbate does not make a major contribution to the antioxidant capacity of blueberry samples tested (*4*).

Kay and Holub (*14*) demonstrated that dietary supplementation with freeze-dried wild blueberry powder increased serum antioxidant status following consumption of a high-fat meal in a study done with a group of eight male human subjects. Increasing serum antioxidant status has been suggested as a possible means of reducing the risk of many chronic degenerative disorders

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(*15,16*). The blueberry treatment was associated with a significantly ($p<0.05$) higher increase in serum antioxidant status relative to the control at 1 and 4 h post consumption on the high-fat meal. Peak serum anthocyanin concentration occurred 60-70 min after consumption (*14*). Furthermore, plasma ORAC peaked at 1 hour in the blueberry treatment group which was significantly different from that of the control group. Kay and Holub (*14*) suggested that anthocyanins together with other phenolic compounds within the blueberry may be regenerating oxidized lipid soluble antioxidants in the serum, similar to the process of vitamin E regeneration by vitamin C (*17*). Furthermore, it has been shown that ascorbate makes very little contribution (0.8 to 1.5%) to the total *in vitro* antioxidant capacity of blueberries (*4,5*).

A multitude of *in vitro* studies has shown that flavonoids can inhibit, and sometimes induce a large variety of enzyme systems that regulate cell division and proliferation of platelet aggregation, detoxification and inflammatory and immune response (*18*). Therefore, dietary flavonoids are believed to control the various stages in cancer process, on the immune systems and in homeostasis in cell systems. It has also been hypothesized that the antioxidant properties of flavonoids may protect tissues against oxygen free radicals and lipid peroxidation (*19*), which involved in several pathological conditions such as atheroscelrosis, cancer and chronic inflammation (*20*). Oxidation of low density lipoprotein (LDL) is thought to play an important role in atherosclerosis. Flavonoids (*21-23*) or food extracts rich in flavonoids (*24,25*) have shown to protect action against *in vitro* LDL-oxidation.

Anthocyanins are natural colorants belonging to the flavonoid family. They are widely distributed among flowers, fruits, especially berries, and vegetables and are responsible for the bright colors such as orange, red and blue. The anthocyanins are glycosides and acylglycosides of anthocyanidins. There are over 250 naturally occurring anthocyanins (*26*). Apart from their function as antioxidants, anthocyanins offer a number of other health benefits. Ballinger *et al.* (*27*) characterized 14 anthocyanins in blueberry fruit. The major anthocyanins were malvidin 3-galactoside, delphinidin 3-galactoside, delphinidin 3-arabinoside, petunidin 3-galactoside, petunidin 3-arabinoside and malvidin 3-arabinoside. Also present in minor amounts were 3-monoglucosides of delphinidin, petunidin, malvidin, peonidin and cyanidin; peonidin 3-arabinoside; and cyanidin 3-galactoside. The total anthocyanin content, which is believed to be controlled by one or two major genes, varied from 25 to 495 mg/100g (*28,29*). Highbush blueberries have been reported to contain 25 to 495 mg/ 100g anthocyanins (*3*). Kalt and McDonald (*30*) reported that lowbush blueberries contained 138 mg anthocyanins per 100 g. Cultivars Tiflue and Bluegreem of Rabbiteye blueberry contained 210 and 270 mg of anthocyains per 100 g, respectively (*31*). Blueberries native to Europe (*V. myrtillus* L.) had the highest anthocyanin content of 300-698 mg/ 100 g (*3*). Anthocyanin content of

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Reveille blueberries ranged from 62 to100 mg/g while that of bilberry was 300 mg/g (*4*).

Anthocyanins have been used to treat various microcirculation diseases resulting from capillary fragility (*32*), treatment of diabetic retinopathy (*33*), fibrocystic disease of breast (*34*). Anthocyanins have also been used as antineoplastic agents (*35*), radiation-protection agents (*36*), vasotonic agents (*37*), vasoprotective and anti-inflammatory agents (*38*), chemoprotective agents against platinum toxicity in anticancer therapy (*39*) and hepatoprotective agents against carbon tetrachloride damage (*40*). The biological effects of anthocyanins might contribute to the beneficial effects of consumption of fruit and vegetables (*41*).

Compared with flavones, anthocyanidins are less active, and this may be attributed to the lack of C-4 carbonyl that, in conjugation with the C-2: C-3 double bond, plays an important role in the efficiency of antioxidants (*41*). Presence of a hydroxyl group at the 5' position does not enhance antioxidant activity, contrary to other flavonoids (*41*). The sugar moiety attached to the flavylium cation seems to influence antioxidant activity, in general, which may account for the difference in their radical scavenging activity (*22,41*). The difference in molecular structure of sugars may cause changes in the structural configuration of anthocyanins that enhance or diminish their ability to form a stable radical (*19*). Thus, presence of glucose enhanced activity to a greater degree than that of rhamnoglucose or galactose (*41*).

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Antioxidant Activity of Other Berries

The antioxidant activity against LDL oxidation was associated directly with anthocyanins and indirectly with flavonols; in liposomes it correlated with hydroxycinnamate content (*42*). Constantino *et al.* (*43*) indicated that the activities of soft black raspberries, black currants, highbush blueberries, blackberries, redcurrants and red raspberries toward chemically generated superoxide radicals were greater than those expected on the basis of anthocyanins and polyphenols present in them. Spray dried eldberry (*Sambucus nigra*) juice containing high amounts of anthocyanin glucosides, showed greater inhibition for copper-induced LDL oxidation (*44*). Furthermore anthocyanins were able to reduce α-tocopheroxyl radical to α-tocopherol. Haibo *et al.* (*45*) reported that anthocyanin and its aglycone, cyanidin isolated from tart cherries (*Prunus cerasus*) were responsible for their antioxidant action. Haibo *et al.* (*46*) revealed that 6,7-dimethoxy 5,8,4'-trihydroxyflavone was the most active compound followed by genistein, chlorogenic acid, naringenin and genistin. Red

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berries in the family Roosaceae, cloudberries (*Rubus idaeus*) and strawberries (*Fragaria spp.*) were especially rich in ellagitannins (ETs); the complex polyphenols classified as hydrolysable tannins (*47*). As ellagitannins are esters of hexahydrodiphenolic acid and glucose or quinic acid, their hydrolysis during extraction or processing treatment, or in the gut, yields ellagic acid (EA). Thus, EA is a compound derived from ellagitannins, and is rarely found in living plants (*48*). Raspberry ellagitannis showed slight inhibition of formation of methyl linoleate hydroperoxide at 50 ppm level and a moderate response at 250 ppm level (*49*). However, cloudberry ellagitannis, showed antioxidant activity at 50 ppm, but none at 250 ppm level. DPPH radical scavenging activity of ellagitannins was extremely high whereas EA showed considerably lower activity at both concentrations (50 and 250 ppm). Landbo and Meyer (*50*) reported that enzymatic maceration of black currant increased the antioxidant activity of the resulting juice. The concentration of anthocyanin was significantly influenced by the particle size of the crushed berries, thus the degree of berry crushing was a main factor for anthocyanin concentration. Furthermore, the anthocyanin concentration tended to increase with increased enzyme dose and increased maceration temperature, but the effects of these parameters as well as the influence of the maceration time varied depending on the enzyme type used for maceration. Kahkonen *et al.* (*49*) investigated the anthocyanin activity of bilberry (*V. mytillus*), cowberry (*V. vitis-idaea*) and black currant (*Ribes nigrum*) extracted with 50% acetonitrile containing 0.5% trifluoroacetic acid. Among berries studied, bilberry was found to be the richest source of total anthocyanins (498 mg/100g fruit weight expressed as cyanidin 3-glucoside equivalents) as well as the number of different anthocyanins identified (15 different molecules). Correspondingly, the total amount of anthocyanins was 237 mg/100g fruit (containing 4 main molecules), and 59mg/100g in cowberries (3 main molecules). Furthermore, the activity of anthocyanidins in preventing the oxidation of emulsified methyl linoleate decreased in the order of malvidin> delphinidin > cyanidin > peonidin > petunidin > pelargonidin. Among the anthocyanidins tested, delphinidin possessed the highest radical scavenging activity, followed by cyanidin, peonidin, pelargonidin, malvidin and petunidin. All three anthocyanin isolates exhibited remarkable inhibition of formation of methyl linoleate hydroperoxide at both 100 and 250 μM concentrations. Black currant anthocyanins showed the highest radical scavenging potential against DPPH radical, followed by bilberry and cowberry (*49*). Wang and Jaio (*5*) reported that 100g of blackberry juice (fresh weight) had a scavenging capacity equal to 3.78 mg of ascorbic acid for hydrogen peroxide, 11.0 mg of glutathione for superoxide radical, 5.47 mg of α-tocopherol for singlet oxygen, 2.4 mg of β-carotene for O_2, and 19.1 mg of chlorogenic acid for hydroxyl radicals.

Use of Luminescence Technique to Evaluate Antioxidant Activity of Blueberries

Blueberry samples from different locations (see Table II) were delivered to the laboratory in the frozen state. Fifty grams of frozen blueberry were homogenized and mixed with water (1:1, w/v). The slurry was centrifuged at 4000g for 5 min. The supernatant (juice fraction) was transferred to vials and stored at –80 C, and then used for determination of total phenolic content as explained by Singleton and Rossi (52). Total content of extracted phenolics was expressed as mg of (+) catechin equivalents per 100 g of fresh fruit.

The radical scavenging capacity of the water extracts was measured by Photochem™. For determination of radical scavenging capacity, 300 μL of fruit extracts were diluted to 10 mL and used for the assay. Ten microliters of the assay sample were mixed with 2.29 mL of ACL Reagent 1® (diluent), 200 μL of ACL Reagent 2® (buffer). These were acquired from Analytik Jena AG (Delware, OH). Twenty five microliters of the working solution of Reagent 3® (luminol) (Reagent 3® was diluted with 750 μL of ACL Reagent 1® to prepare the working solution) were added to the above mixture and vortexed immediately before the measurement. The sample mixture was introduced to the Photochem™ (Analytik Jena AG, Delware, OH) cell and the remaining luminescence after 3 min. was recorded.

The total pehnolics content of blueberries tested ranged from 84 to 278 mg/100g of fresh berries in the water extracts (Table II). Radicals generated photochemically by ultraviolet radiation of a photosensitizer were allowed to be scavenged by samples containing the antioxidant extracts. The remaining radical concentration was quantified by luminescence generation as a result of a chemical reaction with a detection reagent in the detection unit housed in the instrument. The strength of the antioxidative extracts was quantified on the basis of their inhibitory effects on luminescence by comparing with a Trolox standard.

The total phenolic content in berries from Mont Valin (Quebec) was 84 mg/100g fresh fruit while it was present at 278 mg/100g fresh weight for farmed berries from Avalon peninsula, NL. Antioxidative capacity of the berry extracts as measured by Photochem™ followed the same pattern as total phenolic content for all samples examined. The highest inhibition (75%) was shown by farmed berries from Avalon peninsula, Newfoundland while the lowest inhibition (36%) was shown by berries from Mont Valin, Quebec. The relationship between total phenolic content and radical scavenging capacity is depicted in Figure 1. A fairly strong relationship (r = 0.84) existed between the total phenolic content and the radical scavenging activity as measured by Photochem™. Thus, Photochem™ offers a facile method for determination of antioxidant potential of berries and other food constituents. In addition, results of this work show clear

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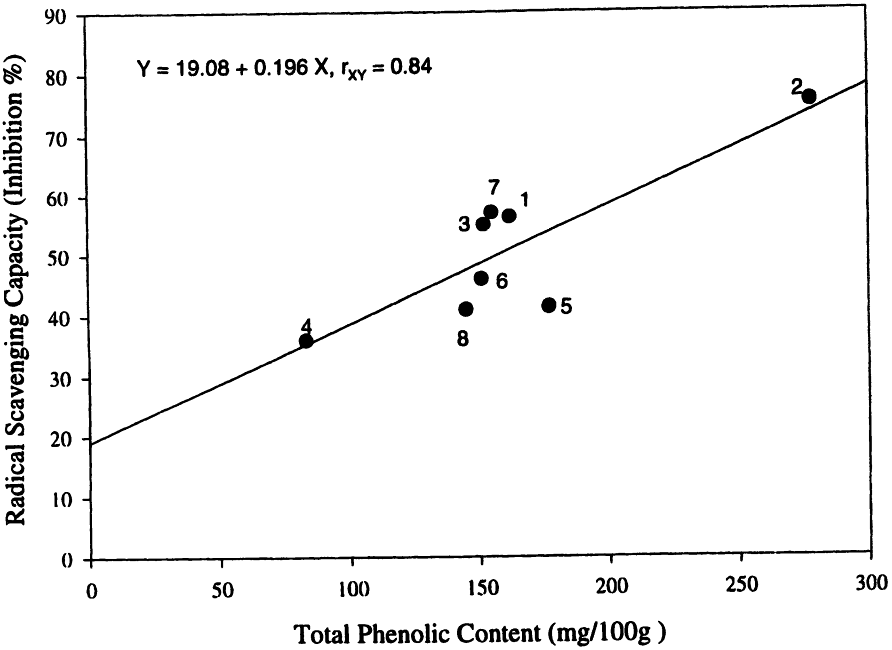

Figure 1. Relationship between total phenolic content (X) (mg/100g) and radical scavenging capacity (inhibition percentage) as measured by Photochem^{TM}.

difference in the content of total phenolics and their antiradical activity based on location of cultivation.

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Chapter 13

Aroma Changes in Green Tea Beverage during Processing and Storage

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The effects of different treatments on aroma changes in green tea beverage during processing and storage were studied. To prepare the green tea beverages, the steamed green tea leaves were further dried for 30 min at various temperatures (a control, 120, 140, and 160 °C), and than extracted with water for 10 min at 60 °C. The extracts were pasteurized for 8 min at 120 °C, and stored at 50 °C to accelerate the storage conditions. To compare the aroma changes caused by various pasteurization methods, some of the extracts were also heat processed at 115 and 125 °C with various durations. The aroma changes of such treated extracts during heating and storage were evaluated by sensory methods. Some selected volatile compounds that are important for tea aromas were also analyzed using solid-phase microextraction-gas chromatography (SPME-GC) method.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Introduction

Since the appearance of Ready-To-Drink (RTD) tea beverages in 1980's, their popularity has been growing and until today, they have become one of the most important beverages in the marketplace. In Japan, the business of tea beverages increased by 21% annually during the end of 1990's, and the market magnitude has reached two million tons in 2000. In China, the current tea beverage business is growing 300% yearly (at present), and it has become the third biggest beverage in China only after carbonated drinks and pure (bottled) water. In the USA, more than 80% of the 90,000 tons of teas consumed every year is finally processed into "iced tea", and bottled or canned iced tea beverages can be found in the vending machines across the country.

Recently, consumers' demand for green tea beverage is increasing due to the revelation of various pharmaceutical functions of green tea. Because of its delicate sensory characters, green tea has also been recommended as the proper material for production of low or sugar-free tea beverages (*1*). On the other hand, manufacturers have noticed that the production of green tea beverage is technologically more difficult than that of black or Oolong teas, mainly due to the instability of its flavor and color. During processing and storage, green tea extract not only changes its color and taste easily (*2*), but aroma, another important sensory attribute of teas, is also difficult to preserve. Both manufacturers and consumers have experienced the aroma differences between the canned or bottled green tea beverages and the freshly brewed green tea. However, this aroma problem has not been effectively improved due to lack of research information. The aroma description of green tea beverage, and the factors that attribute to the aroma changes during its processing and storage have also not been investigated in detail. The objective of this research was to understand the effects of heat processing and storage on the aroma composition and sensory qualities of green tea beverage.

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Materials and Methods

Tea leaves (*Camellia sinensis*), harvested in August 2000 at Cheju island of Korea, were directly processed into steamed green tea (*2*) without further classification. The crude green tea which contained tea leaves of various sizes and stems was vacuum packaged in aluminum-polypropylene bags and stored at −40°C until used.

Sample preparation

(A) Effect of heat processing and storage on green tea beverage

Crude green tea was extracted with 50 times of deionized water (by weight) at 60°C for 10 min. After the tea dregs were removed by successively filtering through 106 μm testing sieve, No.4 and No. 42 Whatman filter paper, the fresh tea extract (sample F) was heat processed at 120°C for 8 min (sample P), cooled to room temperature in a cold water bath, then stored at 50°C to accelerate chemical reactions for up to 6 and 12 days (samples PS6 and PS12, respectively). All samples were transferred to 250 mL polyethylene bottles (green colored), sealed and frozen until analyzed.

(B) Influence of advanced tea leaf roasting on the aroma qualities of green tea beverage

Roasting tea leaves to enhance the aroma is a common method used to improve the flavors of tea extracts. Crude green tea was further roasted at 120, 140 and 160 °C for 30 min, and fresh extracts of these green teas (designated 120F, 140F and 160F, respectively) were prepared as explained in method (A). Comparisons of the flavor qualities among the four unprocessed samples (original F, 120F, 140F and 160F) were conducted.

(C) Changes of aroma qualities of green tea beverage under various heat processing conditions

The HTST (High temperature short time) is a usual technique used for processing of foods and beverages. Since the heat processing conditions vary according to the manufacturer, information of the aroma changes in green tea beverage under various heat processing conditions could be useful reference for manufacturers' processing design.

Fresh green tea extract (F) described in method (A) was processed under three conditions of the equal lethal rate for *Clostridium botulinum* (Z value = 18): 115 °C for 25 min (-5P), 120 °C for 8 min (P), and 125 °C for 2.5 min (+5P). These processed green tea extracts were compared for their aroma composition and sensory qualities.

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Analysis of aroma composition

(A) Solid phase microextraction (SPME)-GC analysis

Seven milliliters of green tea extract were placed into a 10 mL vial and 1.5 g of sodium chloride were added for saturating the extract. One microliter of 0.03% ethyl heptanoate, which has not been reported to occur in tea volatiles, was introduced into the sample as an internal standard. A SPME fiber (75 μm Carboxen-PDMS) (Supelco, Inc., Bellefonte, PA) was exposed to the sample headspace for sampling the volatile compounds at 40 °C (water bath) while the extract was continuously stirred for 40 min. After demoisturization by silica blue for 5 min, the volatile compounds were desorbed by inserting the fiber into a GC injector in splitless mode connected with a fused-silica capillary column (DB-1 Wax, 30m, 0.53 mm ID, 1.5 μm film thickness) (J&W Scientific, Folsom, CA) for 10 min. The initial temperature of GC was set at 40°C for 2 min, then the GC temperature was increased at a rate of 5°C/min until reached to 220°C. GC was operated at this final temperature for another 7 minutes. Quantity changes of individual chemical substances were observed by comparing their peak areas.

A series of hydrocarbon mixture (GL Sciences, Inc., Tokyo, Japan) containing carbons from C_4 to C_{20} was also GC analyzed, and the retention time was used for calculating Kovat's indices of the unknowns (*3*).

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(B) GC-MS analysis

A GC (HP 6890) coupled with a mass spectrometer (HP 5973, Hewlett-Packard, Palo Alto, GA) was used. Except a different column (HP-1, 30 meter, 0.32mm ID and 0.25 μm film thickness) was applied, the GC operation conditions (temperature and time) were the same as described above. The mass spectrometer was operated in the electron ionization (EI) mode at an ionization voltage of 70 eV, in the mass range of 50-550 a.m.u., at a scan rate of 1.53 scan/sec, and at a manifold temperature of 280 °C.

Identification of the volatile compounds was carried out by comparing the mass spectral data with those of the Wiley library, Kovat's indices, and referencing to the previous publications (*4,5*).

Sensory evaluation

Nine trained panelists, composed of 5 males and 4 females, were selected from 30 volunteers. Eleven sensory attributes, including "cucumber", "cooked spinach", "hay", wet woody", "caramel sweet", "chestnut peel", "roasted barley", "citrus", "black tea", "oxidized oil" and "alcohol", were determined by panelists during a triplicated training using green tea beverage samples F and PS12. Prepared samples, after melt from freeze, were placed in colored wine glasses to mask their color, and the top of wine glasses was covered with a piece of watch glass to prevent aroma evaporation. Based on the method of magnitude estimation of descriptive analysis, the questionnaire was composed of lines of 8 cm-the highest intensity was expressed as 8 and the lowest as 0. Triplication of sensory evaluation was conducted under room temperature, and the results were analyzed using ANOVA for multiple comparison (SAS/STAT software, SAS institute Inc., Cary, NC.).

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Results and Discussion

(A) Aroma changes in green tea beverage caused by heat processing and storage

Figure 1 shows the GC chromatograms of aroma compounds detected in samples F, P, and PS12, and their tentative identifications are listed in Table 1. Originally, there were around 40~50 peaks in the GC chromatogram of sample F. After heat processing (sample P), several volatiles, such as *p*-xylene, linalool, ethyl benzoate, decanol, and α-humulene disappeared, while some other new volatiles, including propionaldehyde, n-propanol, isoamyl alcohol, 3-methyl-2-pentanol, 2,2,6-trimethylcyclohexanone, 2,6-dimethyl-hept-5-en-1-al, n-decanal, geraniol, and n-butyl n-butyrate, were formed. During storage, most of these new volatiles continuously increased, and the appearance of more other new compounds (mainly ketones and esters with some alcohols, hydrocarbons, sulfur compounds, lactones, oxygen-containing heterocyclic compounds and free acids) made the GC chromatogram of sample PS12 much more complex (Totally, 76 aroma compounds could be detected). Usually, compounds with shorter retention time could be more important for the aroma of green tea beverage, because their volatilities are lower and closer to the real consumption temperatures. Therefore besides the newly appearing volatiles, several peaks in the front part of chromatogram whose peak areas were significantly increased, such as 2,3-butanedione, n-hexenal, *cis*-3-hexen-1-ol, and n-nonanal, have to be paid more attention.

The changes of aroma compounds during processing and storage contributed to the changes of sensory characteristics in green tea beverage. Figure 2 shows the results of sensory evaluation for samples F, P, PS6, and PS12. After heat processing, the flavors of "cucumber" and "cooked spinach" were greatly reduced, while "wet woody", "caramel sweet", "chestnut peel",

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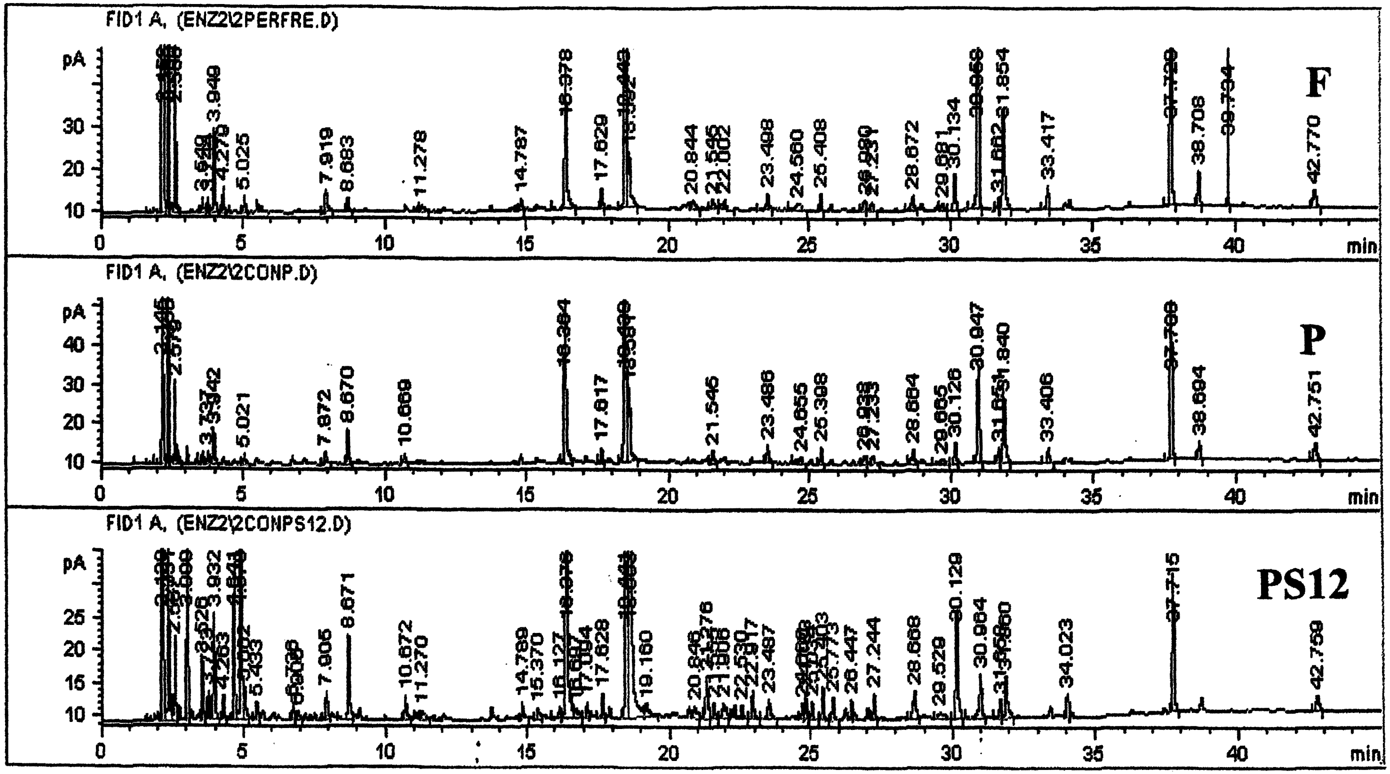

Figure 1. Changes of volatile compounds in green tea beverage by heat processing and storage. (F: fresh extract, P: heat processed at 121°C for 1 minute, PS12: heat processed and stored at 50°C for 12 days)

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Table I. Tentative identification of the peaks in GC chromatograms of Figure 1. "*" indicates the existence of volatile compounds.

Time	KI DB-1	F	P	PS12	Compounds
2.144	430	*	*	*	Carbon dioxide
2.280	454			*	Ethanol
2.349	465	*	*	*	Chloroform
2.443	480		*	*	Porpionaldehyde
2.578	501	*	*	*	2-Methyl-n-propan-2-ol
2.738	515	*	*	*	Methyl acetate
3.015	538		*	*	n-Propanol
3.153	548			*	Methyl vinyl ketone
3.539	576	*	*	*	2,3-Butanedione
3.735	589	*	*	*	Isopropyl ether
3.819	594			*	Ethyl acetate
3.948	602	*	*	*	n-Hexane
4.276	619	*	*	*	2-Methyl-1-propanol
4.657	637			*	Tetrahydrofuran
4.894	647			*	Isopropyl acetate
5.012	653	*	*	*	n-Butanol
5.455	670	*	*	*	3-Methylbutan-2-ol
6.734	718		*	*	Isoamyl alcohol
6.925	725.			*	Methyl isobutyl ketone
7.862	756		*		3-Methyl-2-pentanol
7.921	758	*	*	*	Toluene (Methylbenzene)
8.093	764			*	4-Methyl-3-penten-2-one
8.670	781	*	*	*	n-Hexanal
9.059	792			*	n-Butyl acetate
10.670	844	*	*	*	*cis*-3-Hexen-1-ol
11.137	858	*			Xylene (1,2-Dimethylbenzene)
11.270	862	*		*	Styrene (Ethenylbenzene)
13.693	934			*	Benzaldehyde
14.787	968	*	*	*	6-Methyl-5-hepten-2-one
15.367	984	*	*	*	n-Octanol or 2,3,5-trimethyl-pyrazine

Table I. *Continued.*

Time	KI DB-1	F	P	PS12	Compounds
16.126	1007		*	*	Benzyl alcohol
16.375	1016	*	*	*	2-Ethyl-1-hexanol
16.697	1027			*	*cis*-Ocimene, or β-Phelladrene
17.094	1040		*	*	2,6-Dimethyl-hept-5-en-1-al
17.628	1057	*	*	*	γ-Terpinene
17.864	1065			*	*cis*-Linalool oxide
18.300	1078			*	Methyl benzoate
18.441	1082	ISTD	ISTD	ISTD	Ethyl-n-heptanoate
18.593	1087	*	*	*	Linalool
19.021	1100			*	n-Undecane
19.159	1105			*	2-Isobutyl-4-ethyl-5-methyloxazole
20.525	1153	*			Ethyl benzoate
20.656	1158	*	*	*	Epoxylinalol
21.171	1175			*	Terpinene-4-ol
21.276	1179		*	*	Decamethyl-cyclopentasiloxane
21.366	1181		*		n-Decanal
21.553	1187	*	*	*	β-Cyclocitral
21.997	1202	*	*	*	Dodecane
22.280	1213			*	α-Terpinolene
22.529	1223			*	α-Fenchene
22.917	1237		*	*	Geraniol
23.486	1258	*	*	*	Indole
24.558	1296	*			Decanol
24.666	1300	*	*	*	Tridecane
24.783	1304			*	Theaspirane B
25.034	1315		*	*	n-Butyl n-butyrate
25.403	1329	*	*	*	Dodecamethyl-cyclohexasiloxane
25.773	1344	*	*	*	β-Damascenone

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Table I. *Continued.*

Time	KI DB-1	F	P	PS12	Compounds
26.447	1370			*	Propanoic acid, 2-methyl, 2,2-dimethyl-1-(2-hydroxy -1-methylethyl)propyl ester
27.007	1390	*	*	*	*cis*-Jasmone
27.245	1400	*	*	*	β-Damascone
28.590	1456	*	*	*	3,7-Dimethyl-1,6-Octadiene
28.676	1459	*	*	*	Tetradecane
28.979	1472	*			α-Humulene
29.687	1500	*	*		n-Dodecanol
30.129	1520	*	*	*	2,6-Ditert-butylquinone
30.964	1556	*	*	*	2,6-Di(t-butyl)-4-hydroxy-4-methyl-2,5-cyclohexadien-1-one
31.658	1586	*	*	*	β-Ionone
31.860	1594	*	*	*	Butylated hydroxytoluene (BHT)
33.416	1666	*	*	*	Pentadecane
34.023	1693	*	*	*	Ethyl-2-(4,4-dimethyl-2-methylenecyclopentanyl)acetate
37.716	1874	*	*	*	1,2-Benzenedicarboxylic acid, diethyl ester
38.701	1921	*	*	*	Hexandecane
42.760		*	*	*	Dibutyl phthalate

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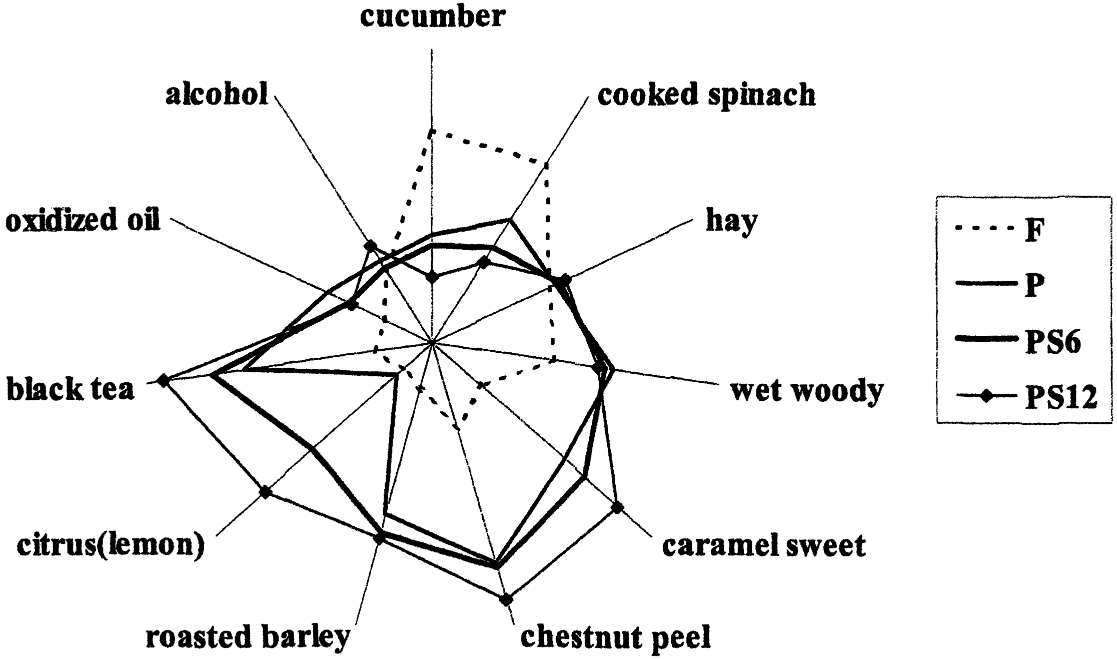

Figure 2. Effect of heat processing and storage on the sensory qualities of green tea beverage. (F: fresh extract, P: heat processed at 121°C for 1 minute, PS6 and PS12: heat processed and stored at 50°C for 6 and 12 days)

"roasted barley", "black tea", and "oxidized oil" were significantly increased. During 12 days of storage, "cucumber" and "cooked spinach" were further decreased, and the intensity of "caramel sweet", "citrus' and "black tea" flavors in sample PS12 were significantly raised compared to sample P. The smells of "hay" and "alcohol" were not much influenced by heat processing and storage.

(B) Effect of advanced tea leaf roasting on the aroma qualities of green tea beverage

The major consideration for the storage of tea leaves is to prevent moisture absorption. Exceeded moisture content not only could cause the growth of microorganisms, but also results in the chemical reactions for the undesirable changes of flavors. Many green tea experts would like to roast their tea leaves stored for a long time before infusion to recover some of the flavors. Also, low-grade green teas, such as ban-cha, are sometimes further roasted to produce a more acceptable grade. The roasting temperatures differ individually, and for some heavily roasted green teas, such as hoji-cha, their roasting temperature could even reach 180 °C (*5*).

Lack of flavors is one of the common problems in products of green tea beverages. To evaluate the effect of advanced tea leaf roasting on the aroma qualities of green tea beverage, tea leaves were treated at three different temperatures (120, 140 and 160 °C) before extraction. Figure 3 and Table 2 show the GC chromatograms and their tentative identifications of fresh green tea extracts (without retort processing) made from these tea leaves. Few differences could be found between the control sample (sample F) and sample 120F (data not shown), and the results of sensory evaluation (Figure 4) also indicated that roasting green tea leaves at 120°C for 30 min didn't significantly enhance the aroma of tea extract. However, the volatiles in samples 140F and 160F were about 1.5 and 2 times of those in the control F, respectively. Most of the new volatiles produced by roasting were the products of Maillard reaction, such as furans, pyrroles, and pyrazines. In addition, the increases of some esters and aldehydes were also observed. These volatiles contributed a lot of "caramel sweet", "chestnut peel", "roasted barley", "black tea" and "oxidized oil" flavors to the green tea extracts, but the aromas of "hay", "wet woody", "citrus", and "alcohol" were almost not changed. In samples 140F and 160F, the "cucumber" and "cooked spinach" flavors were also significantly reduced. They could be just masked by the strong roasting flavor of the newly appearing compounds, because there were no obvious decreases in some alcohols or aldehydes which are the major sources for the greenish aroma in green tea (*6*). It was noted that heavy roasting tea leaves may not be suitable for the manufacture of cold green

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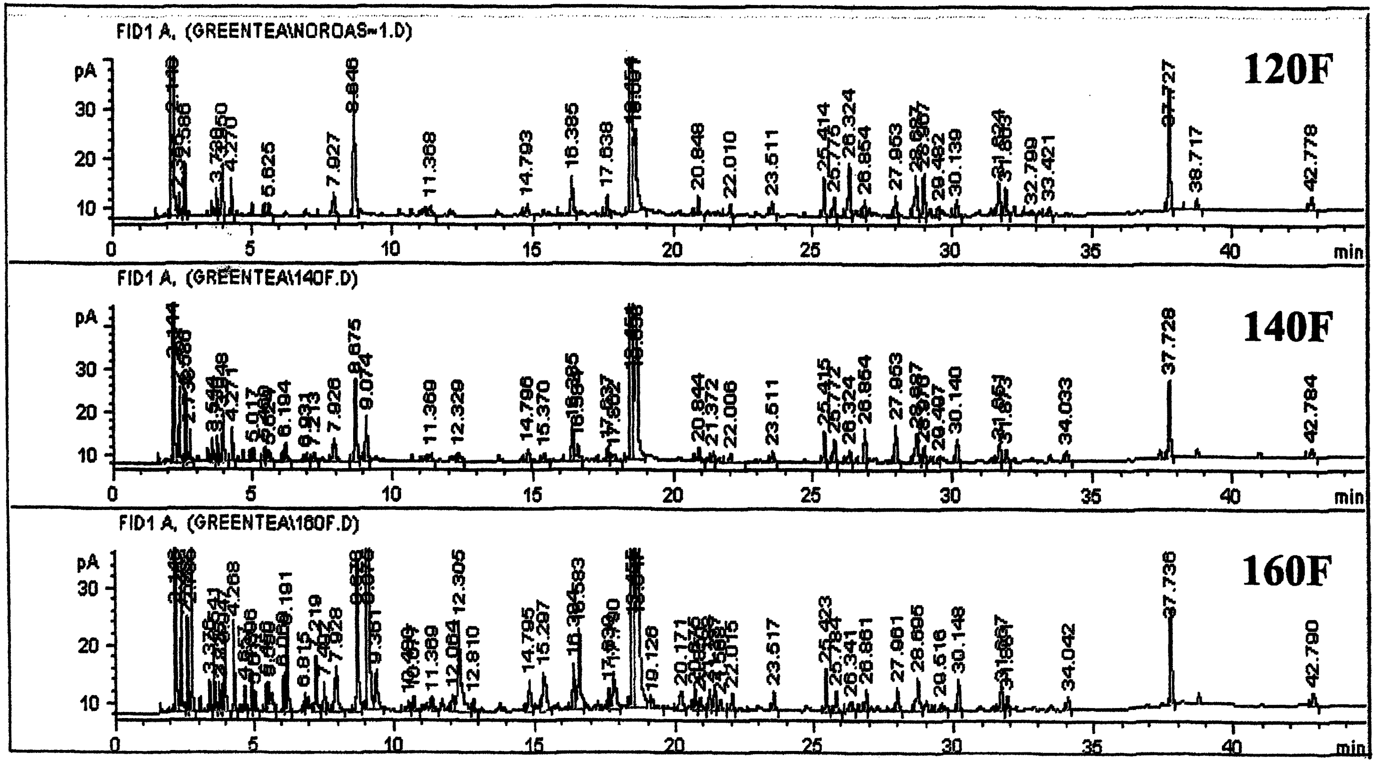

Figure 3. Changes of volatile compounds in green tea beverage by advanced tea leaf roasting. (120F, 140F, and 160F represent the fresh green tea extracts prepared from the tea leaves further roasted at 120°C, 140°C, and 160°C for 30 minutes, respectively.)

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Table II. Tentative identification of the peaks in GC chromatograms of Figure 3. "*" indicates the existence of volatile compounds.

Time	KI DB-1	120F	140F	160F	Compounds
2.146	430	*	*	*	Carbon dioxide
2.304	454	*	*	*	Ethanol
2.356	465	*	*	*	Chloroform
2.450	480	*	*	*	Propinonaldehyde
2.493	488		*	*	Furan
2.587	501	*	*	*	2-Methyl-n-propan-2-ol
2.734	515	*	*	*	Methyl acetate
3.023	538		*	*	n-Propanol
3.374	565	*	*	*	tert-Butyl methyl ether
3.539	576	*	*	*	2,3-Butanedione
3.729	589	*	*	*	Isopropyl ether
3.825	594		*	*	Ethyl acetate
3.946	602	*	*	*	n-Hexane
4.267	619	*	*	*	2-Methyl-1-propanol
4.656	637	*	*	*	Tetrahydrofuran
4.896	647		*	*	Isopropyl acetate
5.016	653	*	*	*	n-Butanol
5.457	670	*	*	*	3-Methylbutan-2-ol
5.550	634		*	*	2-Methyltetrahydrofuran
5.624	677	*	*	*	Cyclohexane
6.069	693		*	*	2-Ethylfurane
6.191	697		*	*	2,5-Dimethylfuran
6.815	721			*	n-Methylpyrrole
6.927	725	*	*	*	Methyl isobutyl ketone
7.050	729			*	Methyl disulfide
7.220	735		*	*	Pyrazine
7.492	744		*	*	Vinyl butyrate
7.593	748			*	Piperidine
7.858	756	*	*	*	3-Methyl-2-pentanol
7.928	758	*	*	*	Toluene

Table II. *Continued.*

Time	KI DB-1	120F	140F	160F	Compounds
8.677	781	*	*	*	n-Hexanal
9.074	792		*	*	1-Ethyl-1H-pyrrol
9.358	800			*	2,5-Dimethyl-1H-pyrrol
10.478	837			*	Methyl pyrazine
10.676	844	*	*	*	*cis*-3-Hexen-1-ol
10.999	853			*	2-Ethyl-1-butanol
11.134	858	*		*	Xylene
11.283	862	*	*	*	Styrene
11.682	864			*	2,5-Dimethylthiophene
12.064	873	*	*	*	Cyclohexone
12.231	884			*	2-n-Butylfuran
12.304	889		*	*	2,5-Dimethyl-pyrazine
12.414	893			*	n-Amyl acetate
12.545	897			*	2,6-Dimethyl-pyrazine
12.810	905			*	Methyl-n-hexanoate
13.712	934		*	*	Benzaldehyde
14.695	964		*	*	Benzonitrile
14.794	968	*	*	*	6-Methyl-5-hepten-2-one
14.998	974			*	2-Ethyl-6-methyl-pyrazine
15.167	979			*	2-Ethyl-3-methyl-pyrazine
15.372	984	*	*	*	2,2,6-Trimethyl-cyclohexanone
15.825	997			*	1-H-Pyrrol-2-carboxaldehyde
16.384	1016	*	*	*	2-Ethyl-1-hexanol
16.497	1020	*	*		1-Undecanol
16.582	1023		*	*	2-Ethyl-3,5-dimethyl-pyrazine
17.235	1044			*	α-Isophorone
17.638	1057	*	*	*	γ-Terpinene

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Table II. *Continued.*

Time	KI DB-1	120F	140F	160F	Compounds
17.789	1062		*	*	2,5-Dimethyl-3-ethylpyrazine
17.867	1065		*	*	2-Ethyl-4,5-dimethylthiazole
18.312	1078			*	2-Butanoylfuran
18.455	1082	ISTD	ISTD	ISTD	Ethyl-n-heptanoate
18.638	1087	*	*	*	Linalool
19.025	1100			*	n-Undecane
19.126	1105			*	2-Isobutyl-4-ethyl-5-methyloxazole
20.174	1141			*	2,3-Diethyl-5,6-dimethylpyrazine
20.548	1154			*	Tetrahydrofurfuryl propionate
20.667	1158		*		Epoxylinalol
20.675	1158			*	3,5-Diethyl-2-methylpyrazine
20.851	1165	*	*	*	Menthol
21.188	1175		*	*	2-Pentanoylfuran or Methyl salicylate
21.378	1181		*	*	n-Decanal
21.568	1187		*	*	β-Cyclocitral
22.015	1202	*	*	*	Dodecane
23.517	1258	*	*	*	Indole
25.423	1329	*	*	*	Dodecamethylcyclohexasiloxane
25.784	1344	*	*	*	β-Damascenone
26.257	1361			*	Acetaldehyde linalyl ethyl acetal
26.341	1366	*	*	*	α-Muurolene
26.574	1374			*	Phenylethyl isobutyrate
26.861	1385	*	*	*	Naphthalene

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Table II. *Continued.*

Time	KI DB-1	120F	140F	160F	Compounds
27.961	1430	*	*	*	δ-Cadinene
28.607	1456	*		*	3,7-Dimethyl-1,6-Octadiene
28.695	1459	*	*	*	Tetradecane
28.983	1472	*	*	*	α-Humulene
29.518	1493		*	*	Geranyl acetone
30.149	1520	*	*	*	2,6-Ditert-butylquinone
31.668	1586	*	*	*	β-Ionone
31.88	1594	*	*	*	Butylated hydroxytoluene
33.422	1666		*		Pentadecane
34.023	1693		*	*	Ethyl-2-(4,4-dimethyl-2-methylenecyclopentanyl)acetate
37.398	1858		*		1,2-Diethyl-3,4,7-trimethoxyindene
37.736	1874	*	*	*	1,2-Benzenedicarboxylic acid, diethyl ester
38.727	1921	*	*	*	Hexandecane
42.760		*	*	*	Dibutyl phthalate

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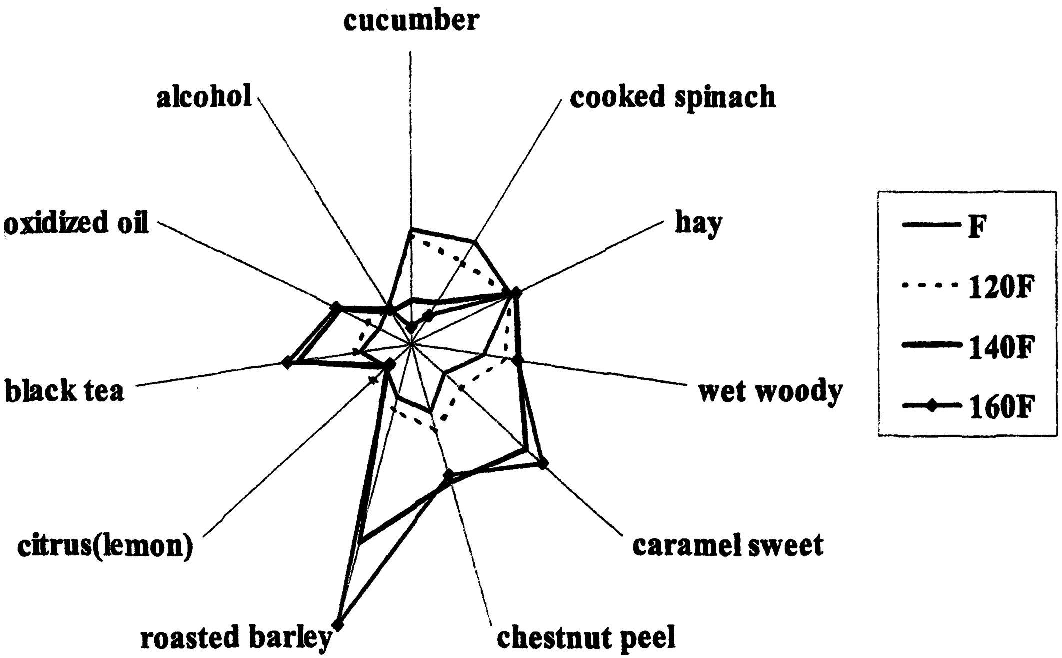

Figure 4. Effect of advanced tea leaf roasting on the aroma qualities of green tea beverages. (F is the control sample extracted from the tea leaves without advanced roasting; 120F, 140F, and 160F represent the fresh green tea extracts prepared from the tea leaves further roasted at 120°C, 140°C, and 160°C for 30 minutes, respectively.)

tea beverage, because the roasting aroma would convert to a kind of non-fresh greasy smell under room temperature.

(C) Influence of heat processing conditions on the aroma characteristics of green tea beverage

From the result of experiment (A), significant differences could be observed in the aromas of green tea beverages before (sample F) and after (sample P) heat processing. Many manufacturers of green tea beverages have also noticed that retort is one of the key steps to determine the final qualities of their products. Huge investment may be required for just a little improvement on the equipment of heat processing. Therefore, how the conditions of heat processing influence the sensory qualities of green tea beverage is necessary to be understood.

Figure 5 shows the comparison among the GC chromatograms of samples processed at three different conditions : 115 °C for 25 min (sample –5P), 120 °C for 8 min (sample P) and 125 °C for 2.5 min (sample +5P). The aroma compositions in sample P and sample +5P were quite similar, but the GC chromatogram for sample –5P was very complex so that more than 150 peaks were detected. In sample –5P, significant increases occurred especially in aldehydes (e.g. isobutyl aldehyde, n-hexanal, n-heptanal, 3,5,5-trimethy-hexanal, etc.), alcohols (e.g. n-butanol, trans-2-hexenol, n-heptan-2-ol, 2-ethyl-1-hexanol etc.), ketones (e.g. 2,3-butanedione, methyl isobutyl ketone, etc.), and esters (e.g. isoamyl acetate, cis-3-hexenyl-formate etc.). In addition, the increase of many hydrocarbon compounds (e.g. n-hexane, toluene, 1,3,5-trimethylbenzene, phellandrene, n-undecane, etc.) could be also meaningful for the aroma changes of sample –5P (The tentative identification for the GC analysis of sample –5P is listed in Table 3).

Well-trained panelists could easily tell the differences between samples –5P and +5P (Figure 6). Extension in processing time significantly increased the “wet woody”, “caramel sweet”, “chestnut peel”, and “black tea” flavors (comparison between samples P and –5P), and bigger gap of processing time (comparison between samples +5P and –5P) could even enlarge the differences in the flavors of “cucumber”, “hay”, and “roasted barley”. Even though no significant difference between samples P and +5P was detected by GC, human beings’ noses seem more sensitive and can smell their difference in “wet woody”. Unlike the changes caused by storage (experiment (A)), heating process didn’t increase the “citrus” flavor in green tea extracts, but increase the smell of “wet woody”.

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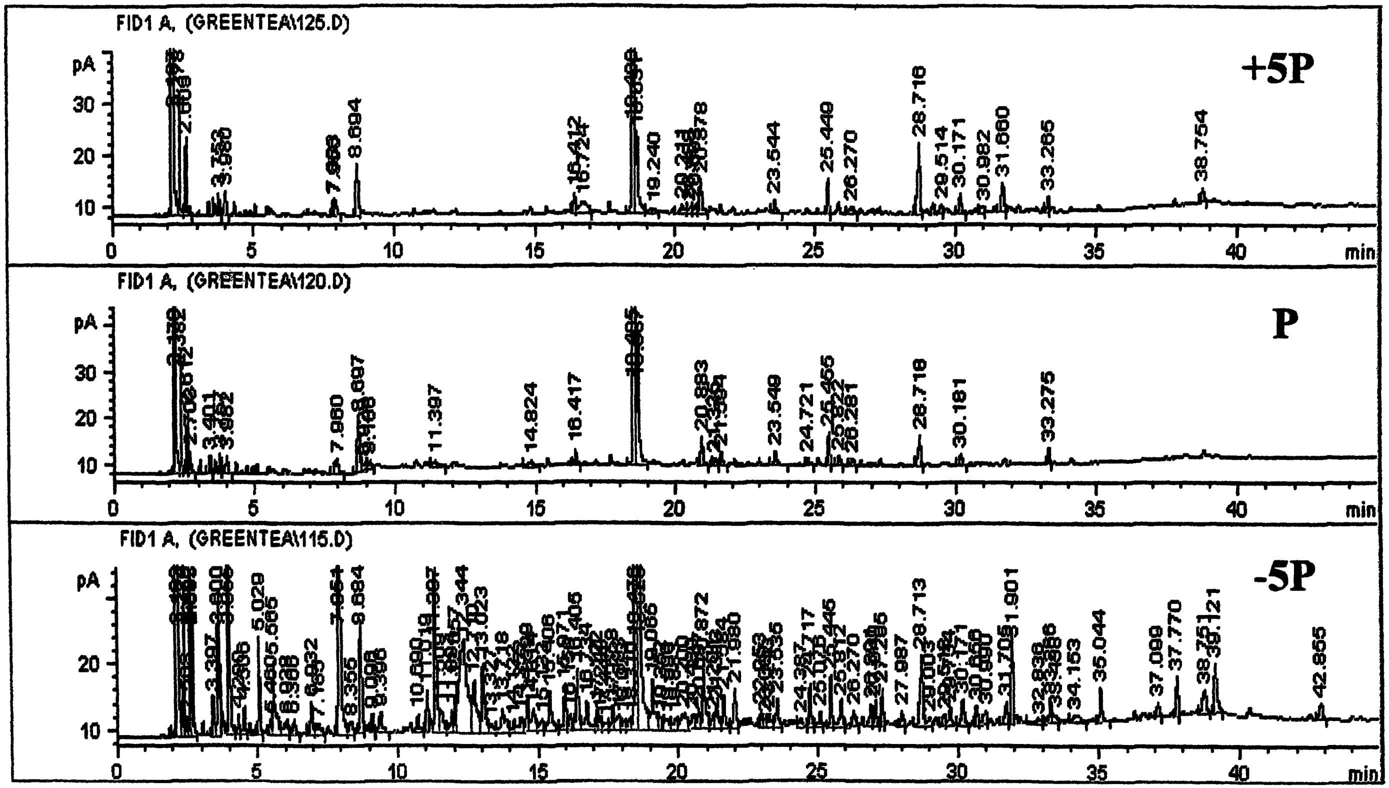

Figure 5. Changes of volatile compounds in green tea beverage by various heat processing conditions. (+5P: heat processed at 120°C for 2.5 minutes; P: heat processed at 120°C for 8 minutes; -5P: heat processed at 115°C for 25 minutes.)

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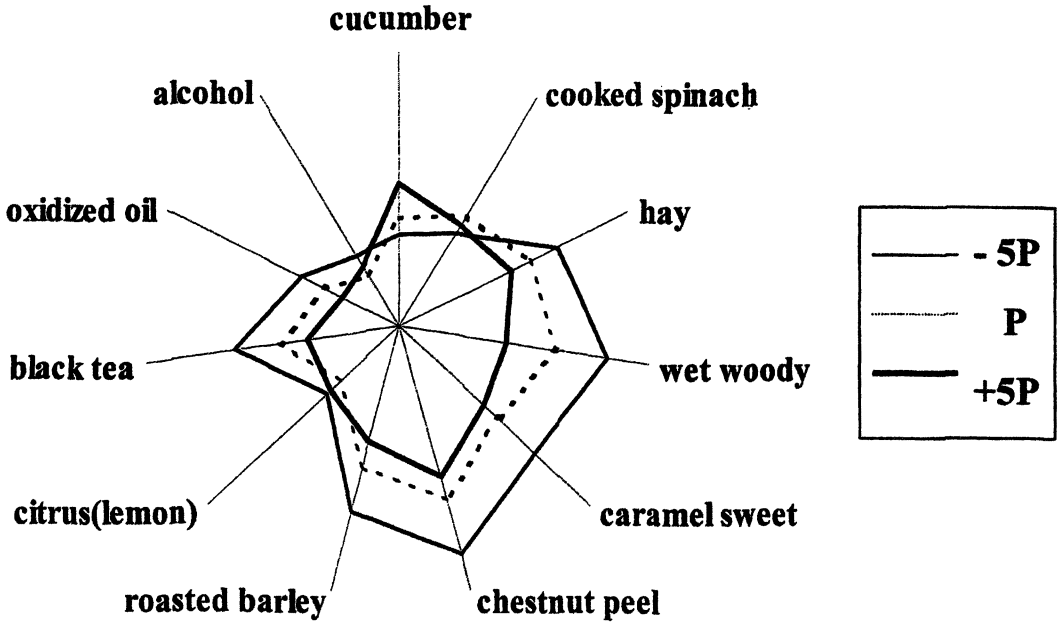

Figure 6. Effect of various heat processing conditions on the aroma qualities of green tea beverages. (+5P: heat processed at 120°C for 2.5 minutes; P: heat processed at 120°C for 8 minutes; -5P: heat processed at 115°C for 25 minutes.)

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Table III. Tentative identification of the peaks in the chromatograms of Figure 5. "*" indicates the existence of volatile compounds.

Time	KI DB-1	-5P	P	+5P	
1.874		*			
2.115	425	*		*	
2.162	433	*	*	*	Carbon dioxide
2.266	451	*	*	*	Ethanol
2.319	460	*		*	1,1'-Oxybis-ethane
2.372	469	*	*	*	Chloroform
2.462	483	*	*	*	Propinaldehyde
2.510	491	*	*	*	2-Methyl-n-propan-2-ol
2.601	503	*	*	*	Isobutyl aldehyde
2.693	511	*	*	*	tert-Butanol
2.756	517	*	*	*	Methyl acetate
3.038	540	*	*		n-Propanol
3.391	566	*	*	*	Diethyl ether
3.425	568	*			Isopropyl formate
3.563	577	*	*	*	2,3-Butanedione
3.610	581	*			n-Butanone
3.658	584	*			Allyl formate
3.745	589	*	*	*	Isopropyl ether
3.843	595	*			Ethyl acetate
3.954	602	*	*	*	n-Hexane
4.290	619	*	*	*	2-Methyl-1-propanol
4.506	630	*			tert-Amyl alcohol
4.674	637	*	*		Tetrahydrofuran
4.914	648	*	*		Isopropyl acetate
5.029	653	*	*	*	n-Butanol
5.464	671	*	*	*	3-Methylbutan-2-ol
5.563	674	*		*	2-Methyltetrahydrofuran
5.644	677	*		*	Cyclohexane
5.709	680	*			Methyl pyruvate
5.959	689	*			Tetrahydropyran or

Table III. *Continued.*

Time	KI DB-1	-5P	P	+5P	
					Diethyl sulfide
6.038	692	*	*		Ethyl n-propionate
6.306	701	*			n-Heptane
6.752	718	*			Isoamyl alcohol
6.932	725	*	*	*	Methyl isobutyl ketone
7.095	731	*			Methyl disulfide
7.186	734	*			Isopropyl n-propionate
7.511	745	*			Vinyl butyrate
7.885	757		*	*	3-Methyl-2-pentanol
7.950	759	*	*	*	Toluene
8.357	772	*			Methyl n-butyl ketone
8.547	777	*			3,4-Hexanedione
8.684	781	*	*	*	n-Hexanal
8.916	788	*	*		4-Methyl-3-Penten-2-one
9.096	793	*			n-Butyl acetate
9.315	799	*			4-Methylthiazole
9.396	801	*			Hexamethyl-cyclotrisiloxane
10.440	836	*			Ethyl 2-methylbutyrate
10.689	844	*			Ethylbenzene
10.705	845		*	*	*cis*-3-Hexenol
11.019	845	*			*trans*-2-Hexenol
11.142	858	*			*p*-Xylene
11.305	863	*			Isoamyl acetate
11.509	868	*			Allyl n-butyrate
11.792	876	*			Di-n-butyl-ether
11.894	879	*			n-Heptan-4-ol
12.056	884	*			n-Heptanal
12.195	888	*			2-Heptan-2-ol
12.346	892	*			Amyl acetate
12.710	902	*			*cis*-3-Hexenyl formate

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Table III. *Continued.*

Time	KI DB-1	-5P	P	+5P	
13.021	912	*			2-Ethyl-4,5-dimethyloxazole
13.320	922	*			Ethyl tiglate
13.718	935	*			1,3,5-Trimethylbenzene
13.811	938	*			α-Thujene
13.870	940	*			Isobutyl n-butyrate
14.121	948	*			Benzaldehyde
14.351	955	*			n-Amyl n-propionate
14.424	957	*			2-Ethyl-hexanal
14.600	962	*			3,5,5-Trimethyl-n-hexanal
14.814	968	*	*	*	6-Methyl-5-hepten-2-one
14.920	972	*			Methyl n-hexyl ketone
15.134	978	*			n-Butyl n-butyrate
15.266	982	*			Ethyl-n-hexanoate
15.405	986	*	*	*	n-Octanol or 2,3,5-Trimethyl-pyrazine
15.971	1002	*			α-Phellandrene
16.149	1008	*			2,2,6-Trimethylcyclohexanone
16.292	1013	*			n-Hexyl acetate
16.404	1018	*	*	*	2-Ethyl-1-hexanol
16.733	1025	*		*	*cis*-Ocimene, or β-Phelladrene
17.122	1039	*			2,6-Dimethyl-hept-5-en-1-al
17.243	1045	*			*trans*-2-Octenal
17.368	1049	*			Methyl hexyl acetaldehyde
17.657	1058	*	*	*	γ-Terpinene
17.810	1063	*			n-Octanol
17.884	1065	*			2-Ethyl-4,5-dimethylthiazole
18.022	1070	*			*m*-Diethylbenzene
18.203	1075	*			5-Nonanone
18.314	1079	*			Methyl benzoate
18.476	1084	ISTD	ISTD	ISTD	Ethyl-n-heptanoate
18.627	1088	*	*	*	Linalool

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Table III. *Continued.*

Time	KI DB-1	-5P	P	+5P	
19.065	1101	*			n-Undecane
19.179	1106	*			2-Isobutyl-4-ethyl-5-methyloxazole
19.352	1112	*			Norbornyl acetate
19.468	1116	*			Methyl 2-hydroxyisobutyrate
19.590	1121	*			Dihydrolinalool
19.879	1131	*			Cyclooctanol
20.105	1139	*			Dihydrocinnamic aldehyde
20.199	1142	*			1,3-Dimethoxy benzene
20.283	1145	*			1,4-Dimethoxy benzene
20.489	1152	*			Diethyl succinate
20.561	1155	*			Ethyl benzoate
20.696	1159	*	*	*	Epoxylinalol
20.872	1165	*	*	*	Menthol
21.030	1170	*			Terpinolene
21.202	1176	*			Decamethyl-cyclopentasiloxane
21.313	1180	*	*		n-Decanal
21.583	1189	*	*	*	β-Cyclocitral
21.980	1202	*			Dodecane
22.048	1204		*	*	Isobutyl disulfide
22.197	1210	*			*p*-tert-Butylphenylpropyl aldehyde 1
22.322	1215	*			n-Amyl tiglate
22.438	1219	*			Ethyl phenylacetate
22.562	1224	*			n-Hexyl 2-methyl butyrate
22.953	1238	*	*		Isoamyl n-hexanoate
23.087	1243	*			Geraniol
23.242	1249	*			Geranonitrile 3
23.535	1260	*	*	*	Indole
23.766	1268	*			*o*-Methyoxyacetophenone

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Table III. *Continued.*

	Time	KI DB-1	−5P	P	+5P
24.072	1279	*			Safrole
24.169	1282	*			6−Undecanol
24.387	1290	*			n−Undecanal
24.716	1302	*	*	*	Tridecane
25.077	1316	*	*		n−Butyl n−butyrate
25.445	1331	*	*	*	Dodecamethyl−cyclohexasiloxane
25.813	1345	*	*	*	β−Damasenone
26.269	1363	*	*	*	Isoxazole, 4−(4,5−dihydro−4,4−dimethyl−2−oxazolyl)−5−ethyl−3−methyl
26.353	1366	*	*	*	Propanoic acid, 2−methyl, 2,2−dimethyl−1−(2−hydroxy−1−methylethyl)propyl ester
26.586	1375	*			α−Copaene
26.890	1386	*			Isolongifolene
27.048	1392	*			*cis*−Jasmone
27.296	1402	*	*	*	β−Damascone
27.987	1431	*			α−Gurjunene
28.713	1461	*	*	*	Tetradecane
29.004	1472	*			α−Humulene
29.207	1481	*		*	*Trans*−Caryophellene
29.519	1493	*		*	n−Dodecanol
29.735	1502	*			α−Muurolene or 2−Cyclohexylcyclohexanone
30.171	1521	*	*	*	2,6−Di−tert−butylquinone
30.654	1543	*			Helional or Tangerinal
30.991	1557	*		*	2,6−di(t−butyl)−4−hydroxy−4−methyl−2,5−cyclohexadien−1−one
31.397	1575			*	n−Butyl n−decanoate
31.706	1588	*	*	*	β−Ionone

Table III. *Continued.*

Time	KI DB-1	−5P	P	+5P	
31.902	1596	*		*	Butylated hydroxytoluene (BHT)
32.041	1602	*			Tetradecamethyl cycloheptasilosane
32.202	1610			*	*cis*-3-Hexenyl phenylacetate
33.266	1659	*	*	*	Pentadecane
33.384	1664	*			Propanoic acid, 2-methyl-1-(1,1-dimethylethyl)-2-methyl-1,3-propanediyl ester2-methyl-
34.152	1699	*	*		Ethyl-2-(4,4-dimethyl-2-methylenecyclopentanyl)acetate
35.042	1742	*			Hexanedioic acid, bis(2-methylpropyl) ester
36.297	1802	*			Noot ketone
37.097	1842	*			Phenylethyl benzoate
37.770	1876	*		*	1,2-Benzenedicarboxylic acid, diethyl ester
38.751	1922	*	*	*	Hexandecane
39.120	1939	*			Furaltadone
42.852		*	*	*	Dibutyl phthalate

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Conclusions

SPME-GC analysis accompanied with sensory evaluation could be an efficient method for investigating the aroma problems in the production and storage of green tea beverages. Even though all of the undesirable green tea extracts share the same characteristic in their appearance, that is, the darkened color, more detailed research by checking their various changes in aromas can help decide the problem sources as to whether the tea leaves over-roasted, or improper heat processing methods were applied, or the distribution/storage conditions needed improvement. In addition, the volatile compound appearing right after ethyl heptanoate (the internal standard), tentatively identified as n-nonanal, could be a good index for the freshness of green tea beverage. It could be easily detected and its increase correlates well with all of the situations for heat processing, storage, or tea leaf roasting.

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Chapter 14

Isoflavone Stability in Chocolate Beverages

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This study determined the effect of UHT processing and three storage temperatures (4, 23 and 38°C) on the compositional stability of isoflavone levels in a chocolate flavored high protein beverage product containing isolated soy protein. Multivariate analysis indicated significant changes in isoflavone profile from pre- to post- processing. Univariate analysis of variance showed that shifts occurred within the isoflavone families, but family totals and total isoflavone retention were unaffected. Malonyl conjugates of all three isoflavone families, genistein, daidzein and glycitein decreased significantly, while a significant increase in all unesterified glucosides as well as the acetyl conjugates of daidzin and genistin was observed. Changes within the isoflavone families continued to occur in the ultra high temperature (UHT) processed product during storage. The degree of change was affected mostly by storage temperature, with higher temperatures directly affecting the greatest change. Total isoflavones and totals within an isoflavone family were not reduced over time or by temperature.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

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During the last 20 years, the U.S. food industry has experienced tremendous success in marketing new products with sales of nearly $43 billion to satisfy increasingly health conscious Americans (*1*). 'Reduced Calorie', 'Light', and 'Low Fat' are a few of the claims that the food marketers have used to promote the lack of nutrients on the labels of new products. More recently, the trend is to link the diet and disease relationship to functional foods or nutraceuticals. Rather than avoiding certain nutrients, this new trend takes advantage of the health benefits believed to derive from the phytochemical properties of functional foods.

The term phytochemical refers to every naturally occurring chemical substance produced by plants, especially those substances that are biologically active (*2*). Mounting scientific evidence indicates that a class of phytochemicals found in soy, isoflavones, can lower incidences of human heart disease and cancer, reduce osteoporosis risk, and ease menopausal symptoms (*3-8*). Isoflavones have a structure similar to mammalian estrogens, exhibiting weak estrogenic effects under certain circumstances and anti-estrogenic effects under others. Although the exact mechanisms are not know, it has been postulated that isoflavones can function as estrogen receptors, protease activators, and antioxidants (*9*). Most of the evidence has been provided by epidemiological studies correlating a high consumption of phytoestrogens with a lower incidence of the so-called 'Western' diseased (*10*).

Isoflavone precursors are found in fiber-rich unrefined grain products, various seeds, cereals, and legumes. The major soybean isoflavone aglycones, genistein and daidzein, were identified in the 1940's. Ten additional naturally occurring isoflavones have been identified in soybeans. The 12 soy isoflavone forms are found in three families (genistein, daidzein, and glycitein) and occur as unconjugated isoflavones (aglycones) or as conjugated isoflavones (aglycone glycosides and malonyl and acetyl glycoside esters) (*11,12*). The distribution of these compounds has been documented in various geographically collected soybeans, and in traditional commercial soy foods.

Studies describing the effect of processing treatments on the isoflavone form and content of soyfoods have been limited, generally focusing on soy foods prepared from whole soybeans. The processing methods studied transform soybeans into soy foods such as soy flour, soymilk, miso, tempeh and tofu (*13-15*). Certain types of processing result in isoflavone loss, particularly during processes involving liquids such as extracting, soaking, washing, or boiling when the isoflavones are leached into the liquid and the liquid portion is discarded. Alcohol washing results in more isoflavone losses than water washing. Heat treatment and fermentation tend to modify the profile of the isoflavone forms. Heat treatment tends to decrease malonyl glycoside esters and increase acetyl glycoside esters. The acetyl derivatives could be formed from the corresponding malonyl derivatives during heat treatment (*11*). Conversely, fermentation decreases the isoflavone glycosides with a corresponding increase of the aglycone form.

With expanding technologies, foods containing soy encompass a large array of soy materials, i.e., soy grits, flour, textured flour, concentrate, and isolated soy protein. As foods formulated from these various soy materials become more prominent in the functional foods/nutraceuticals arena, there will be an increasing need for research on the stability of phytochemical components, to help substantiate claims about their stability within the food matrix system during product manufacture and storage. Product developers will be especially interested in the stability of isoflavones under commercial processing and storage conditions, particularly when foods are formulated using one of more of the soy components derived from whole beans. To address these issues, the present study evaluated the changes in the profile of isoflavone components and in isoflavone content, as a result of ultra high temperature (UHT) processing, storage temperature and during storage in a high-protein soy beverage product, formulated with isolated soy protein. Commercial companies have analyzed the isoflavone levels present in isolated soy protein. However, they receive inquiries concerning the effect of processing on isoflavones in products made with isolated soy proteins. Information regarding isoflavone stability in isolated soy proteins is very limited in the scientific literature.

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Materials and Methods

An experimental proprietary chocolate flavored high protein nutritional beverage was formulated by Protein Technologies International, St. Louis, MO. The beverage product was formulated with a blend of isolated soy protein products to provide 25 g of soy protein per 300 mL serving. The isoflavone concentration of the beverage ranged from 1.8 – 2.8 mg of total isoflavones in aglycone units per g of protein.

The product was formulated, processed, and aseptically packaged to maintain a one year shelf life. Hydrolyzed and non-hydrolyzed protein products were blended to achieve the desired finished mouthfeel and beverage viscosity. Additional ingredients were combined in a pre-mix to provide flavoring, assist in protein suspension, and provide nutritional enrichment.

Production of the beverage took place at an equipment manufacturing research center. Each batch of product (1.5 kiloliters) consumed one day's production time. First, 95% of the required water was heated to 74 – 77°C in a 1.5 kL tank. A liquid anti-foam agent was added. All dry ingredients were then added to the water via a Breddo Liquifier (Breddo Likwifier, Kansas City, MO). After 20 min of mixing, a sample was taken for moisture and solids analysis. Any water needed to achieve target formula solids was added. Further mixing (5 min) incorporated the additional water. Temperature of the slurry at the end of mixing was 63 – 65°C. Total mix time was ca. 40 min.

A sample cup was lowered into the batch tank to obtain ca. 100 mL of product. A Mettler HR73 Halogen Moisture Analyzer (Mettler Toledo, Columbus, OH) was used to determine moisture. Solids were calculated by difference after moisture was determined. The settings for the analyzer included a rapid drying pattern at 141°C. This pattern enabled the apparatus to heat very quickly to the temperature setting and then maintain that temperature. This heat pattern prevented the formation of a skin from the beverage sample, which would have trapped moisture.

Just prior to the initiation of thermal processing, a 250 mL sterile bottle was filled with product and refrigerated (4°C). The cold sample was shipped overnight to Ralston Analytical Laboratories (St. Louis, MO) for protein and isoflavone analysis within 24 hr.

A full size process system was used for the UHT process. A combination of Multitube 54/4*16-6, and Monotube TTF 54/25-6 tubular heat exchangers (Alfa Laval, Lund, Sweden) were used. A SHL 20 homogenizer (Alfa Laval, Lund, Sweden) was used for homogenization (175.8 kg/cm^2 1st stage, 35.2 kg/cm^2 2nd stage), post-UHT processing.

The UHT process for the chocolate flavored products consisted of a sterilization temperature of 142°C held for 4.7 s to attain an F_0 of 8.9. The sterilization temperature was kept 2-3 degrees higher than the target temperature to assure the minimum temperature of 142°C was reached. The products were homogenized at 71°C. Product was kept in a sterile surge tank for less than 1 h.

The beverage was packaged in 325 mL cartons on equipment designed to provide an aseptic environment. Products were filled at 15-21°C. A nitrogen flush filled the headspace of the beverage carton. Target weight ranged from 314 – 318g.

Finished packaged products were sampled from the beginning, mid-point and end of the production run. Samples were appropriately labeled and held at 22°C for no longer than 36 h.

A three sample composite was submitted to Ralston Analytical Laboratories for analysis of protein and isoflavone profile. Protein was determined using the standard AOAC method 920.87 (*16*) and the isoflavone profile was determined using a laboratory method developed jointly by Protein Technologies International and Ralston Analytical Laboratories. The method is based on extracting the isoflavones with 80% methanol and a reverse phase HPLC separation with UV detection at 260 nm.

For each of 4 batches of product produced, 108 samples were collected: 3 samples per 3 storage temperatures per month for 12 months. One sample was needed for analysis, and the additional two samples were backup in case of packaging failure. Three environmentally controlled storage were chosen for temperature and humidity: refrigeration 4°C, room temperature 23°C, relative humidity, 50%) and an elevated temperature (38°C, relative humidity, 20%).

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One sample from each batch for each storage condition per month was analyzed for protein content and isoflavone profile. A baseline analysis of the isoflavone profile was conducted for each of the four trials. Subsequent analyses were also conducted for each of 11 months (with the exception of month two) following the production of the beverage products.

The pre-and post-processing effects of time and storage conditions on the isoflavone profile and the total isoflavone content were analyzed for each batch. To remove the time-to-time variation in the analytical method, the analysis of individual components was performed, based on change as a percent of the total for the family. The percent contribution of each conjugate was calculated within the isoflavone family. The change in percent contribution is the difference between the monthly result as compared to the baseline result. Multivariate analysis was used to determine when the point of significant change occurred. After finding significance for the multivariate analysis, a univariate analysis of variance for a completely randomized block design was used to determine which components of the isoflavone profile differed from pre- to post-processing. The profiles were examined in all forms, and percent of the family that was attributed to each member. Significance was reported at $p<0.05$. Results were analyzed in SAS version 6 (SAS Institute, Inc., Cary, NC).

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Results and Discussion

Results from this study indicated that the design parameters (processing, time, and storage temperature) influenced the profile of the isoflavone composition. The data were analyzed from the percent of family contribution perspective. Multivariate analysis of variance was used to determine if the isoflavone profile as a whole changed from pre- to post-processing. A significant change in isoflavone profiles occurred during processing (Table I). Heat processing had a direct affect on the composition of the isoflavone profile within the isoflavone families of daidzein, genistein and glycitin. Figures 1 and 2 highlight the percent contribution changes which occurred within the daidzein and genistein families.

The daidzin and genistin malonyl conjugates decreased significantly due to the de-esterification into the acetyl and glucoside conjugates, after UHT processing. This is consistent with changes observed in studies which included a heat application process (*13,17-19*). There was no significant change in the aglycone forms, daidzein and genistein, as these forms are the most stable (Table I). The thermodynamic stability of these aglycones is due to the delocalization of the π-bond electrons in their conjugated aromatic moieties (*20*). This delocalization does not extend to the glucosidic or higher side-chain groups.

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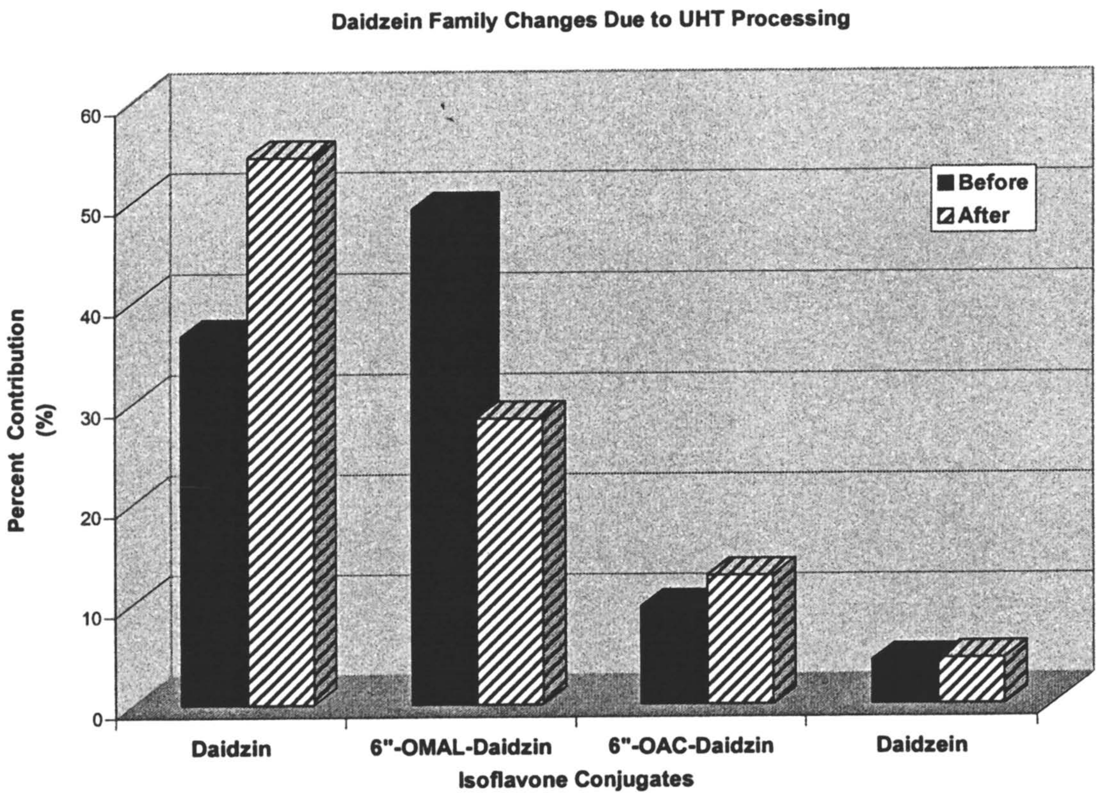

Figure 1. Percent contribution of daidzein isoflavone conjugates before and after UHT processing.

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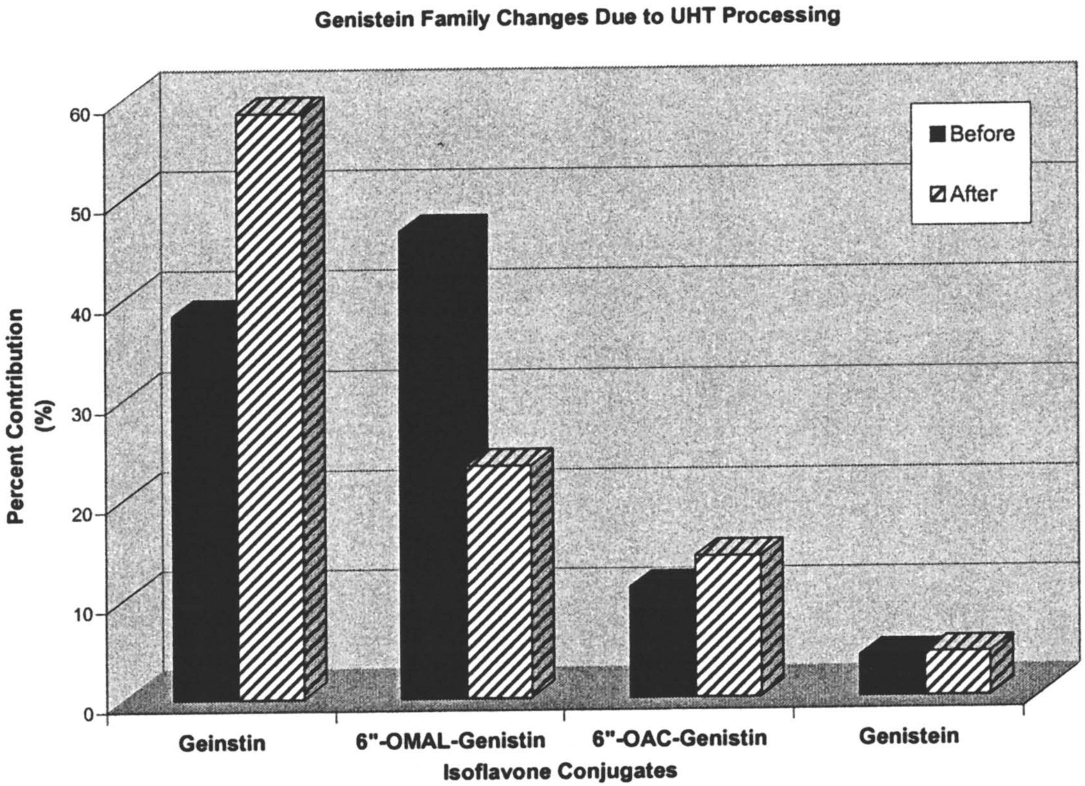

Figure 2. Percent contribution of genistein isoflavone conjugates before and after UHT processing.

With the glycitein family, the significant decrease in 6"-OMAL-glycitin was offset by corresponding increases in glycitin and glycitein. The glycitein family comprised 6.7% of the total isoflavone composition in this study.

Eleven of the known 12 isoflavone compounds were identified and measured. 6"-OAC- glycitin was not detectable. The six aglycones and glucosides were measured against authentic standards, while for the glucoside esters, the glucosides standard curve was used for quantitation based on the assumption that they have the same UV absorption. Concentrations of glucoside esters were adjusted for the molecular weight difference (*19*).

The totals within each family are a sum of both measurements with standards and estimated values based on calculated response factors. Although the profiles within the isoflavone family changed, the total quantity of the isoflavone families and total isoflavones did not change significantly ($p \leq 0.05$) (Table I).

The least stable compounds of the daidzein and genistein families (malonyl and acetyl conjugates) continued to de-esterify into the more stable glucosidic forms (daidzin and genistin) during storage. This change was significant ($p \leq 0.05$) one month after the baseline was established for all storage temperatures. The shifts in the isoflavone profile over time were similar for the daidzein and genistein families during the 11 months.

Although the profile of the isoflavone conjugates within a family initially shifted with storage time, the total isoflavone content was not significantly affected. Data in Table II compare the baseline totals to the average family totals and the total isoflavone content over the 11 month storage time.

While it has been documented that heat during processing directly affects the isoflavone profile (*11,19,21-24*) of soy proteins, this study found that the storage temperature directly affects the magnitude of the change. The changes happened early during storage and were more pronounced at higher storage temperatures.

The apparent pattern of isoflavone composition change is consistent between the daidzein and genistein families with each storage temperature. The glucoside forms increased with time; the acetyl and malonyl conjugates decreased; and the aglycones showed little change. The decrease in 6"-OMAL-daidzin and 6"-OMAL-genistin was significant after one month of storage for all storage temperatures. The change in 6"-OMAL-daidzin and 6"-OMAL-genistin was significantly less when stored at 4°C than at storage temperatures of 23°C or 38°C. The increase in daidzin was significant after one month of storage at 23 or 38°C, and at three months of storage at 4°C. However, the increase for genistin was significant at one month for all temperatures. The change in daidzin and genistin was significantly less when stored at 4°C. The changes in daidzein and genistein were much less than the changes in the other three components of each respective family.

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Table I. Isoflavons in pre- to post- processing (ppm; as is basis)

	All Forms		*Absolute*	
Isoflavones	*Before*	*After*	*Change*	*Change %*
Daidzin	37.2	59.8	22.6	60.8
6"-OMAL-Daidzin	52.5	32.5	(20.0)	(38.1)
6"-OAC-Daidzin	10.3	14.3	4.0	38.8
Daidzein	4.7	5.2	0.5	10.6
Total Daidzein	104.7	111.8	7.1	6.8
Geinstin	59.5	91.7	32.2	54.1
6"-OMAL-Genistin	75	36.8	(38.2)	(50.9)
6"-OAC-Genistin	17.5	22.3	4.8	27.4
Genistein	6.5	6.8	0.3	4.6
Total Genistein	158.5	157.6	(0.9)	(0.6)
Glycitin	7.0	10.2	3.2	45.7
6"-OMAL-Glycitin	10.5	7.7	(2.8)	(26.7)
Glycitein	1.0	1.8	0.8	80.0
Total Glycitein	18.8	19.8	1.0	5.3
Total Isoflavones	282.0	289.2	7.2	2.6

Mean changes that are underlined differ significantly ($p<0.05$) from 0.

Table II. Baseline and total isoflavones (ppm) over time (as is basis).

		All Forms		
Isoflavone Family	*Baseline Total (ppm)*	*4°C Average Total (ppm)*	*23°C Average Total (ppm)*	*38°C Average Total (ppm)*
Daidzein	113.5	113.5	109.3	102.5
Genistein	176.0	194.0	193.0	191.8
Glycitein	23.75	26.35	24.68	23.90
Total Isoflavones	313.2	333.9	327.0	318.2

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The higher the temperature during storage, the shorter the time required for de-esterification from the higher molecular weight conjugates into the glucosidic forms. Barnes *et al.* (*19*) also noted that isoflavones in 80% aqueous methanol extracts of soy materials when stored at room temperature were converted gradually from the 6"-OMAL-glucosidic forms to the β-glucosides.

The trends observed for the retention and distribution of isoflavone conjugates over the storage period indicated that the retention of isoflavones is unaffected, but the distribution of isoflavone conjugates was altered during the first month of storage. The conversion of 6"-OMAL-Glucosides to 6"-OAC-Glucosides and to β-glucosides was similar to the alteration that resulted during the UHT processing. Barnes *et al.* (*19*) observed a gradual change in the composition of isoflavone conjugates when extracts were held during extended periods at room temperature. Consistently, in all three temperatures, the two major isoflavone families, daidzein and genistein, remained stable after the first month. These results emphasize the importance of determining if the bioavailablity of isoflavone conjugates differ.

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Chapter 15

Influence of Melanoidins on the Aroma Staling of Coffee Beverage

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Sensory analysis on aqueous biomimetic coffee aroma recombinates in the absence or presence of coffee melanoidins revealed that, in particular, the intensity of the roasty-sulfury aroma quality was reduced when melanoidins were present. Comparative aroma dilution analysis on the headspaces of aqueous solutions containing the total coffee volatiles, alone, or in mixture with melanoidins revealed that the losses of the odor-active thiols 2-furfurylthiol (FFT), 3-methyl-2-butenthiol, 3-mercapto-3-methylbutyl formate, 2-methyl-3-furanthiol, and methane thiol are responsible for the aroma change. Quantification by means of stable isotope dilution assays confirmed the rapid loss of these thiols during warm-keeping of the coffee brew. Using synthetic [$^2H_2$]-FFT as an example, ^2H NMR and LC/MS experiments gave strong evidence that thiols are covalently bound to the coffee melanoidins via Maillard-derived pyrazinium compounds formed by oxidation of 1,4-bis-(5-amino-5-carboxy-1-pentyl) pyrazinium radical cations (CROSSPY), which were recently identified as key intermediates in roasting-induced coffee melanoidin genesis. Using synthetic 1,4-diethyl diquaternary pyrazinium ions or N_α-acetyl-L-lysine/glycolaldehyde and FFT, it was shown that 2-(2-furyl)methylthio-1,4-dihydro-pyrazines, bis[2-(2-furyl)methylthio]-1,4-dihydro-pyrazines and 2-(2-furyl)methylthio-hydroxy-1,4-dihydro-pyrazines were formed as the primary reaction products. On the basis of these results it can be concluded that the CROSSPY-derived pyrazinium intermediates are involved in the rapid covalent binding of thiols to melanoidins, and, consequently, contribute to the decrease in the sulfury-roasty odor note observed after preparation of the coffee brew.

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The stimulatory, pleasant overall aroma is one of the most important attributes determining the consumer acceptance of a freshly prepared coffee brew. Unfortunately, this desirable aroma is not stable and rapidly changes shortly after preparation of the coffee brew. While numerous studies have addressed the aroma changes during storage of ground coffee powder (e.g. *1,2*), the chemistry responsible for this rapid aroma staling of coffee beverages is as of yet not understood. Recent investigations, combining instrumental analysis with human olfactory perception (e.g. GC/olfactometry), have revealed a rapid decrease in the concentrations of some odor-active thiols when coffee brews were manufactured at elevated temperatures. This is particulary the case in the manufacturing of instant coffee (*3*) as well as during heat sterilization of canned coffee drinks (*4*). Based on model experiments with odor-active disulfides and egg albumin, the decrease of thiols and disulfides in foods was recently suggested to be the result of an interchange with sulfhydryl and disulfide groups of proteins (*5*). It is, however, as yet not clear whether the aroma changes in coffee brews are due to similar reactions involving cysteine residues of the macromolecular coffee pigments, the so-called melanoidins, which are present in concentrations of more than 200 mg per cup of beverage.

The purpose of this investigation was, therefore, (i) to characterize the key coffee odorants affected by melanoidins, and (ii) to elucidate the chemical mechanisms involved in the aroma change occurring during warm-keeping of coffee beverages.

Experimental

Materials

A biomimetic recombinate of coffee brew aroma was prepared using 25 key odorants in their "natural" concentrations (*6*). The 1,4-diethylpyrazinium diquaternary salt was synthesized by perethylation of pyrazine with triethyloxonium tetrafluoroborate (*7*).

Isolation of total volatiles from coffee brew

A coffee brew was prepared by percolation of freshly ground coffee powder (Coffea arabica; var. Caturra; medium roasted) with boiling water (50 g powder/L water). Using the SAFE apparatus developed recently (*8*), an aqueous fraction of the volatiles was isolated from the brew (100 mL) by high-vacuum distillation.

Isolation of melanoidins from coffee brew

Coffee powder (50 g) was extracted with hot tap water (1 L, 80-90°C), the aqueous solution was defatted by dichloromethane extraction and, finally, concentrated by freeze-drying (yield: 12.5 g). Aliquots (1.0 g) of this material, dissolved in water (20 mL), were either fractionated by ultrafiltration (Diaflo PM3, Amicon, Witten) to obtain melanoidins (0.35 g; molecular weight > 3000 Da) after freeze-drying, or separated by gel permeation chromatography on Sephadex G-25 fine (75 x 5 cm i.d.; Pharmacia, Uppsala, Sweden) using water (4 mL/min) as the eluent (*9*).

Static headspace gas chromatography/olfactometry

The aqueous aroma destillate (10 mL) alone, or mixed with coffee melanoidins (125 mg), respectively, was equilibrated in a septum-sealed vessel (240 mL) for 30 min at 40°C. Stepwise decreased headspace volumes (25 to 0.2 mL) were then analysed by headspace-HRGC/olfactometry as recently reported (*9*)

Determination of headspace concentrations of thiols

A solution of 3-methyl-2-buten-1-thiol (400 µg), 2-furfurylthiol (600 µg) and 3-mercapto-3-methylbutylformate (500 µg) dissolved in phosphate buffer (10 mL; 0.1 mmol/L; pH 6.0) alone, or mixed with melanoidins (125 mg), respectively, was equilibrated in a closed vessel (240 mL) at 30°C. The amounts of thiols in the headspace before and after melanoidin addition were determined (*9*).

Quantification of thiols and disulfides by stable isotope dilution analysis

2-Furfurylthiol, 3-mercato-3-methylbutyl formate and bis(2-furfuryl) disulfide were quantified by means of stable isotope dilution analysis in coffee brews either freshly prepared, or kept warm in a thermo flask, and in binary mixtures containing thiols and melanoidins, respectively (*6*).

Spectroscopic measurements

GC/MS was performed using a CP 9001 gas chromatograph (Chrompack, Frankfurt, Germany) equipped with a fused silica capillary CP-WAX 52 CB (25m × 0.32 mm, 1.2 µm film thickness, Chrompack) and coupled with the mass spectrometer Incos XL (Finnigan, Bremen, Germany). LC/MS was performed with a LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). Electron paramagnetic resonance (EPR) and ^{2}H NMR spectra were recorded on an ESP 300 and an AMX 500 spectrometer (Bruker, Rheinstetten, Germany).

Table I. Influence of warm-keeping on aroma quality of a coffee brew

Aroma quality	*Intensity after*[a]		
	0 min	*60 min*	*210 min*
sweet/caramel	1.6	2.1	2.5
earthy	1.9	1.8	1.9
sulfury/roasty	2.3	1.2	0.4
smoky	2.0	2.3	2.3

[a] The intensities of the given odour qualities were scored on a scale from 0 (not detectable) to 3 (strong).

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Results and Discussion

In order to investigate the aroma change of a coffee beverage during warm-keeping, sensory analyses were performed on coffee brews freshly prepared or stored in thermos flasks for 60 or 210 min, respectively (Table I).

A drastic decrease of the intensity of the sulfury-roasty odor quality was observed with increasing storage time, e.g. on a scale from 0 (not detectable) to 3 (strong) the intensity dropped from 2.3 to 0.4 during storing of the brew for 210 min (Table I). In contrast, the intensity of the sweet/caramel-like and the smoky note only increased to some extent, and the earthy aroma quality did not change significantly.

To study whether interactions between the coffee odorants and the melanoidins might be responsible for the aroma change observed, an aqueous aroma recombinate mimicking the overall aroma of an authentic fresh coffee brew was prepared using 25 coffee odorants in their "natural" concentrations (*9*). One aliquot of this recombinate was sensorially evaluated in comparison to an original coffee brew without any further additions (Table II), and the second aliquot was mixed with "natural" amounts of melanoidins (MW>3000 Da), which had been isolated from coffee brew by means of ultrafiltration. In comparison to the aroma profile of a fresh coffee brew and the aroma recombinate, addition of melanoidins reduced, in particular, the intensity of the sulfury-roasty odor quality after an equilibration time of 30 min at 40°C (Table II). These data clearly demonstrate that the melanoidins are somehow involved in degradation or binding of the sulfury/roasty smelling key coffee odorants (*9*).

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Table II. Influence of coffee melanoidins (CM) on the overall aroma of a biomimetic coffee aroma recombinate (CAR)

Aroma quality	*Intensity in*		
	coffee brew	*CAR*[a]	*CAR+CM*[a]
sweet/caramel	1.6	2.1	1.9
earthy	1.9	1.7	1.9
sulfury/roasty	2.3	2.1	1.2
smoky	2.0	1.4	1.6

[a] The aroma profile of an biomimetic coffee aroma recombinate (CAR; 10 mL) was analysed in the absence or presence of coffee melanoidins (CM; 125 mg; MW>3000 Da) after storing for 30 min at 40°C.

To systematically elucidate the roasty-sulfury odorants affected by the coffee melanoidins, the total volatiles and the melanoidins (MW>3000 Da), respectively, were separately isolated from a fresh coffee brew prior to the analytical experiments, and then recombined. In a first experiment, by application of the comparative aroma dilution analysis, the odor-active compounds in a stored model solution (A in Table III) only containing the coffee brew volatiles were compared to those of a second model (B in Table III) containing both, the volatile fraction and the melanoidins in their "natural" concentrations (*10*). The results revealed 16 odorants in the headspace of model A after incubation for 30 min with relative flavor dilution (rFD) factors of 2 to 256 (A in Table III). After incubation with melanoidins (B in Table III), only 15 odorants were detectable, and, in addition, the rFD factors of all thiols were drastically decreased when melanoidins were present (*10*). The most pronounced effects were measured for 2-furfurylthiol (FFT), 3-methyl-2-buten-1-thiol (MBT), and 3-mercapto-3-methylbutyl formate (MMBF), the rFD factors of which were decreased by factors of 16, 8 or 4, respectively (Table III). Also 2-methyl-3-furanthiol (MFT) was significantly decreased, and methane thiol could not be detected at all. In contrast, the aroma impacts of odor-active 2,3-diones, phenols or pyrazines, were not significantly affected upon addition of melanoidins (Table III).

To further confirm this decrease in thiol concentration, the amounts of FFT and MMBF were quantified in coffee brews kept warm in a thermos flask for 0, 30, 60, 90 and 210 min (Figure 1). As given in Figure 1, the freshly prepared coffee beverage contained about 16.0 or 8.2 µg of FFT or MMBF, respectively. Warm-keeping of the brew then led to a drastic decrease in the concentrations of both thiols (*10*). After 60 min the FFT concentration decreased by a factor of more than four compared to the fresh coffee brew. Extending the storage time to 210 min finally resulted in a complete loss of FFT, and only small amounts of MMBF were still detectable (Figure 1). These data, being well in line with the results of the comparative aroma dilution analysis (cf. Table III), clearly demonstrate that the decrease of the sulfury-roasty odor quality observed during storage of coffee brew is mainly due to the loss of odor-active thiols.

Table III. Comparative aroma dilution analysis of the headspace of aroma distillates incubated in the absence (A) or presence (B) of melanoidins[a]

Odorant	*Aroma quality*	*rFD factor*	
		A	*B*
butane-2,3-dione	Buttery	256	128
pentane-2,3-dione	Buttery	128	128
3-methylbutanal	Malty	64	64
2-methylbutanal	Malty	32	64
Acetaldehyde	Fruity	32	32
Methional	potato-like	32	16
2-furfurylthiol	roasty, sulfury	32	2
2-ethyl-3,5-dimethylpyrazine	Earthy	32	32
2,3-diethyl-5-methylpyrazine	Earthy	32	32
Guaiacol	Phenolic	16	32
dimethyl trisulfide	cabbage-like	16	32
2-isobutyl-3-methoxypyrazine	green, earthy	16	16
3-methyl-2-butenthiol	foxy, skunky	8	1
3-mercapto-3-methylbutyl formate	Catty	8	2
2-methyl-3-furanthiol	meat-like	4	2
methane thiol	cabbage-like	2	<1

[a] A high vacuum distillate (10 mL) of a coffee brew, either alone (A), or (B) in mixture with melanoidins (125 mg; MW>3 kDa) were stored for 30 min at 40°C.

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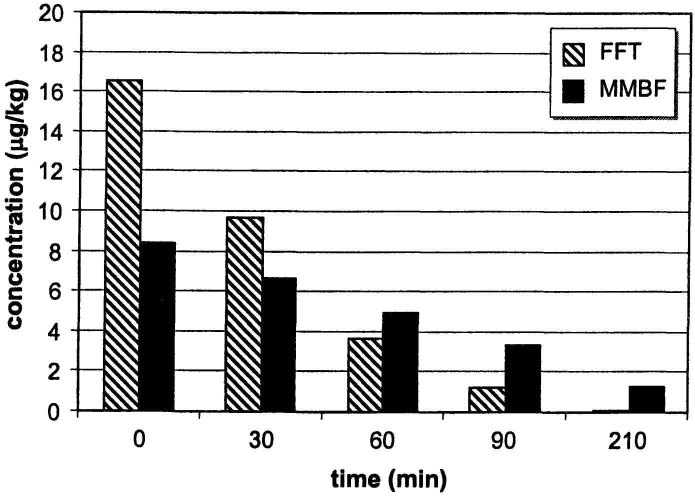

Figure 1. Influence of storage time on the concentrations of 2-furfurylthiol (FFT) and 3-mercapto-3-methylbutyl formate (MMBF) in a coffee brew maintained at 80°C in a thermos flask.

In order to study the mechanisms of the thiol/melanoidin interaction, aqueous solutions of FFT, MMBF, and MBT were incubated either in the absence, or in the presence of coffee melanoidins for 30 min at 30°C (*9*). The decrease in thiol concentration was determined by means of headspace/HRGC by comparing the control (without melanoidins) to the sample with added melanoidins (Figure 2).

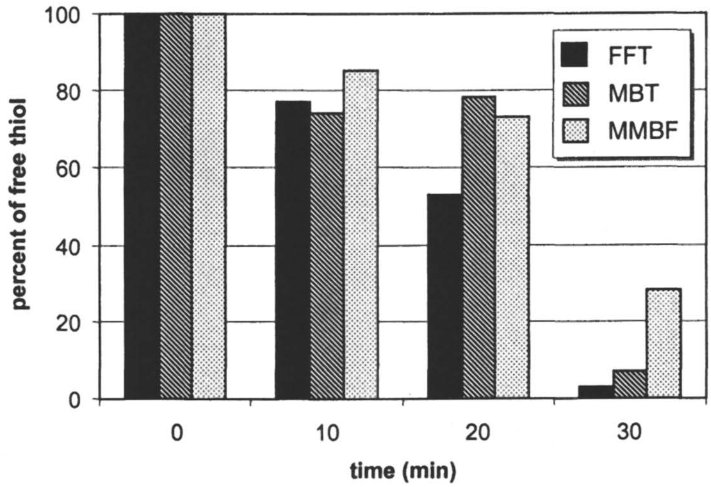

Figure 2. Influence of storage time on the headspace concentrations of thiols in aqueous solutions of 2-furfurylthiol (FFT), 3-mercapto-3-methylbutyl formate (MMBF), 3-methyl-2-buten-1-thiol (MBT), and coffee melanoidins (MW>3000 Da) maintained at 30°C.

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The results revealed that the amounts of each of the three thiols were strongly reduced in the presence of coffee melanoidins and showed that the decrease in concentration proceeds very rapidly (Figure 2), e.g. 50 % of FFT was lost after 20 min. After 30 min, the FFT was nearly absent in the headspace (*9*).

In order to reveal whether these thiols are chemically degraded, or covalently bound to melanoidins, coffee melanoidins were incubated with $[^2H_2]$-2-furfurylthiol ($[^2H_2]$-FFT) for 90 min at 30°C, then freed again from low-molecular weight compounds by ultrafiltration, and, finally, analysed by ^2H-NMR spectroscopy (*10*). As controls, aqueous solutions of coffee melanoidins and $[^2H_2]$-FFT, respectively, were analysed. As displayed in the ^2H-NMR spectrum in Figure 3A, a solution of $[^2H_2]$-FFT in H_2O showed two resonance signals, one at 3.67 ppm corresponding to the deuterated methylene group in the odorant, and another at 4.70 ppm corresponding to the natural ^2H-abundance in tap water. ^2H-NMR of the melanoidins isolated from coffee (Figure 3B) did not show any signals besides the natural ^2H-abundance of the solvent (*10*). Coffee melanoidins, however, which had been pre-incubated with $[^2H_2]$-FFT, showed additional resonance at 3.0-4.2 ppm with a strong line broadening (Figure 3C) as typically found for compounds covalently linked to macromolecules (*10*). These data clearly confirmed the idea that the odor-active thiols are bound to the coffee melanoidins.

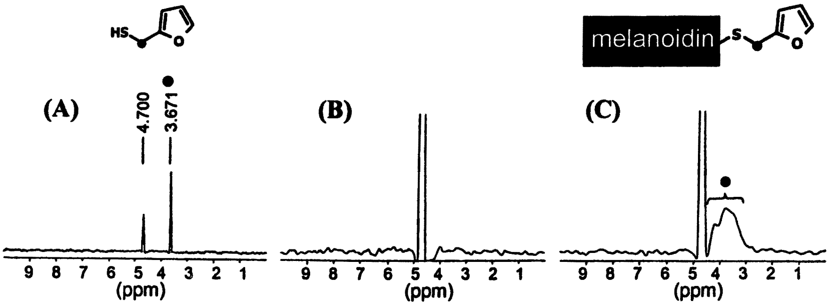

Figure 3. ^2H-NMR spectra (500 MHz, H_2O) of (A) $[^2H_2]$-FFT (1 mg/mL), (B) coffee melanoidins (100 mg/mL), (C) coffee melanoidins (100 mg/mL) after pre-incubation (90 min, 30°C) with $[^2H_2]$-FFT (2 mg) and purification.

Because recent studies had clearly shown that the addition of the reducing agent dithioerythrytol was not able to regenerate major amounts of the "free" thiol from coffee melanoidins, which had been preincubated with FFT (*9*), it can be speculated that the thiols do not bind via disulfide bonds of, e.g. cysteinyl residues, to the coffee melanoidins. Obviously, the thiols preferably react with other reactive sites present in the macromolecules.

Model studies on potential binding sites

To investigate the role of chlorogenic acid moieties present in coffee melanoidins, an aqueous solution of FFT was incubated for 30 min at 30°C either in the presence of free chlorogenic acid, or in the presence of chlorogenic acid, which had been thermally pretreated for 5 min at 230°C to simulate roasting conditions. After incubation with FFT the amounts of the thiol left were analyzed.

Neither untreated (A in Figure 4), nor pre-heated chlorogenic acid (B in Figure 4) showed strong binding activity, since the losses in both models were below 20 per cent (*10*). To study the role of Maillard-derived reaction products in thiol binding, a dry-heated protein/glucose mixture was stored together with FFT. The results showed that the thiol concentration was decreased by a factor of nearly two (C in Figure 4), thus demonstrating that Maillard-derived reaction product's might play a role in thiol binding (*10*). Another experiment using a thermally processed mixture of protein and glycolaldehyde, a carbohydrate cleavage product present in roasted coffee in high amounts, led to a more pronounced effect, because the FFT concentration was reduced to below 30 % (D; Figure 4). Because the ε-amino groups of protein-bound lysine are known as primary targets in Maillard reactions during coffee roasting, the protein was substituted by N_α-acetyl-L-lysine. The dark brown material formed upon roasting was most effective in thiol binding, because only 17 % of the "free" FFT were left in the headspace of the model reaction mixture (E; Figure 4).

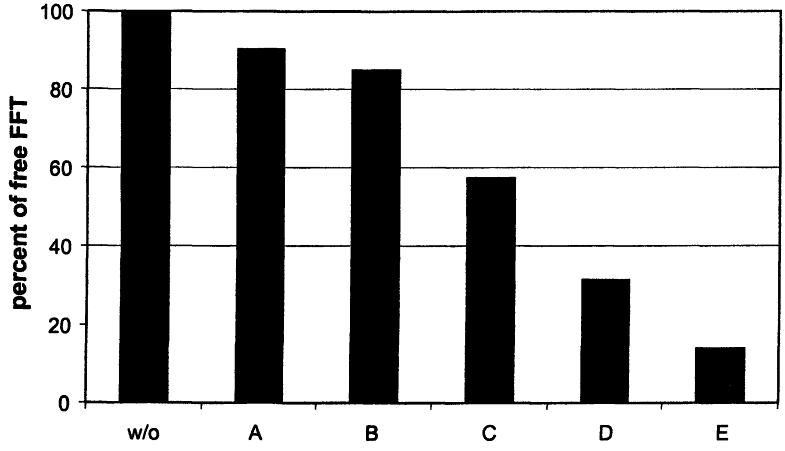

Figure 4. Relative amounts of FFT present in the headspaces of aqueous FFT solutions stored in the presence of (w/o) no additives, (A) chlorogenic acid (20 mg), (B) pre-heated chlorogenic acid (20 mg; 5 min at 230°C), (C) heated albumine/glucose (10 mg each; 5 min at 230°C), (D) glycolaldehyde/albumine (10 mg each; 5 min at 230°C), (E) glycolaldehyde/N_α-acetyl-L-lysine (10 mg each; 5 min at 230°C).

It is well documented in the literature that free radicals are present in roasted coffee (*11-17*), and that 1,4-bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations (CROSSPY), which had been recently shown to be formed from protein-bound lysine side chains and glycolaldehyde, contribute to melanoidin genesis during coffee roasting (*16,17*). In order to gain more detailed insights into the role of radicals as potential thiol binding sites, coffee melanoidins were separated into four fractions by means of gel permeation chromatography (Figure 5, left), and the fractions obtained were investigated for their thiol binding as well as for their free radical activities (Figure 5, right). Incubation of fractions I to IV in the presence of FFT resulted in a complete loss of FFT after 30 min at 30°C (IV; Figure 5). Fraction I and II showed somewhat lower activities, whereas fraction III was least effective in FFT binding (*10*). Analysis of the fractions by means of

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EPR spectroscopy revealed that the radical activities run in parallel with the thiol binding activity, e.g. fraction IV showed the most pronounced effect in thiol binding and exhibited the highest radical activity, whereas fraction III had the lowest potential in thiol binding and the lowest radical activity (Figure 5, right).

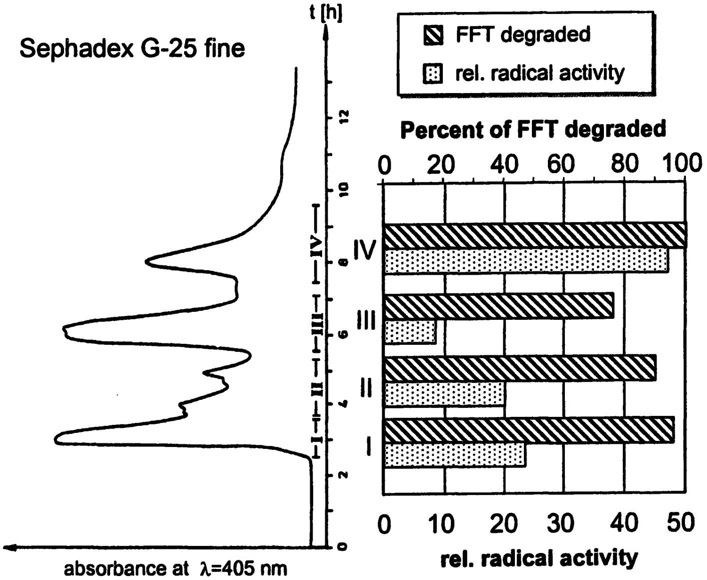

Figure 5. Separation of coffee melanoidins by gel permeation chromatography (GPC; left). Free radical activity in GPC fraction I to IV, and amount of FFT degraded in an aqueous solution of FFT without (w/o), or in the presence of GPC fractions I to IV after 30 min at 30°C (right).

The role of the CROSSPY radical in thiol binding

1,4-Bis-(5-amino-5-carboxy-1-pentyl)pyrazinium radical cations, named CROSSPY (I in Figure 6), were recently identified in melanoidins isolated from coffee brew (*16,17*). These radical cations, found to participate in a redox cycle of reaction intermediates, are oxidized into diquaternary pyrazinium ions (II in Figure 6), which subsequently form 2-hydroxy-1,4-dihydropyrazines (III in Figure 6) upon hydratization, and regenerate the CROSSPY radicals upon a redox reaction with III (*16,17*). Due to their strong browning activity, the bis- and mono-hydroxylated 1,4-dihydropyrazines (IV and III in Figure 6) were recently proposed as penultimate monomers involved in melanoidin genesis (*17*), e.g. by oligomerization reactions via the dimer V (Figure 6).

This redox cycle can be modelled with 1,4-diethyl pyrazinium diquaternary ions (Diquat) as a suitable template to mimic the reactions of lysine-bound pyrazinium derivatives (*17*). Characterization of the reaction products formed upon dissolving Diquat in water by LC/MS spectroscopy (Figure 7) revealed that all reaction intermediates proposed in Figure 6 are generated, namely the CROSSPY-type radical cation (m/z 138), the 2-hydroxy-1,4-diethyl-1,4-dihydropyrazine (m/z = 155), the dihydroxy-1,4-diethyl-1,4-dihydropyrazine (m/z = 171), and the bis-hydroxy dimer (m/z = 309).

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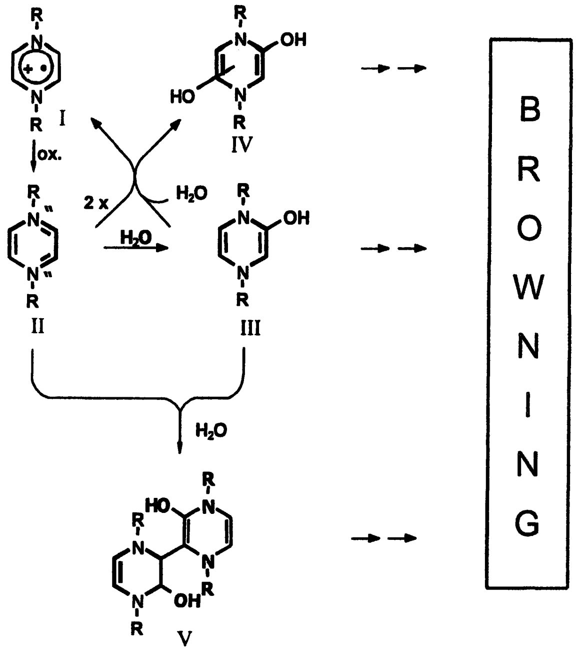

Figure 6. Proposed pathway of melanoidin genesis via CROSSPY (I) and diquaternary pyrazinium ions (II) as the key intermediates (R= protein-bound lysine side chain).

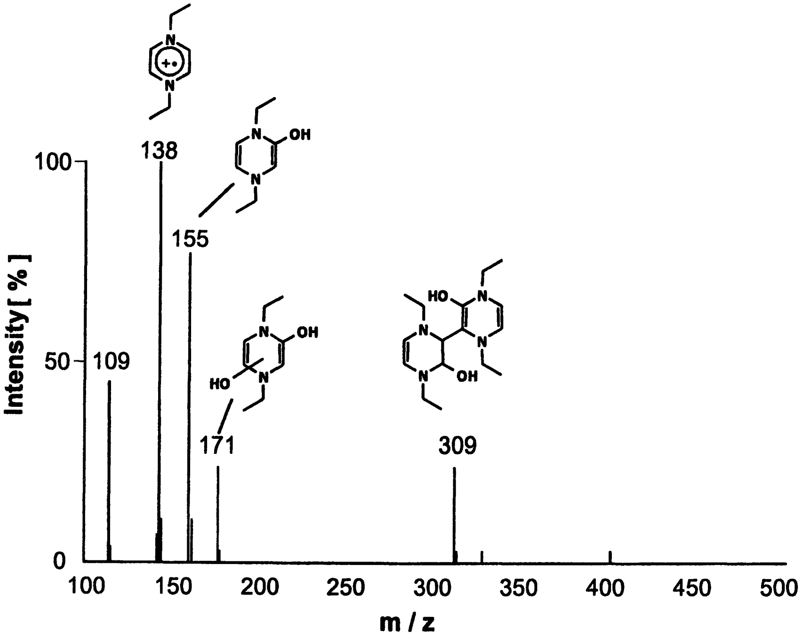

Figure 7. LC/MS spectrum of a solution of 1,4-diethylpyrazine diquaternary ions (Diquat) in water.

To study possible reactions between odor-active thiols and these CROSSPY-associated intermediates, an aqueous solution of Diquat was incubated in the presence of FFT at 30°C. Quantitation of the concentrations of FFT revealed a rapid decrease induced either by the addition of the Diquat solution, or the coffee melanoidins (Figure 8). Both, the Diquat-derived intermediates as well as the coffee melanoidins showed similar kinetics of FFT degradation. Although about 400 or 330 μg FFT, respectively, were bound to the coffee melanoidins or the Diquat-derived intermediates, respectively, less than 6 μg of the corresponding bis(2-furfuryl) disulfide (FFT-S_2) were generated (*10*). These results clearly demonstrate that neither the Diquat solution, nor the coffee melanoidins have the potential to oxidize the thiol into the corresponding disulfide.

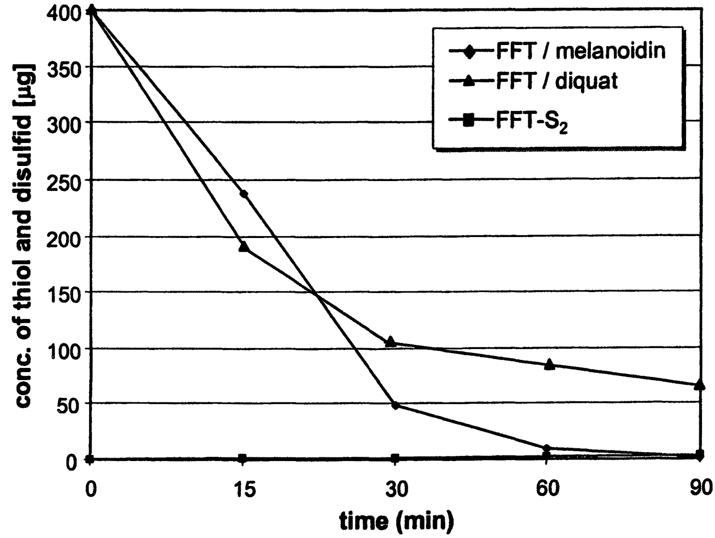

Figure 8. Influence of reaction time on the concentrations of 2-furfurylthiol (FFT) and disulfide (FFT-S_2) in the presence of melanoidins or Diquat-derived reaction intermediates, respectively.

To elucidate the chemical mechanism of thiol binding, an aqueous solution of Diquat was incubated with equimolar amounts of FFT for 10 min at 30°C, and was analysed by LC/MS (Figure 9A). The mass spectrum exhibited a molecular ion at m/z = 251 (100%), which on the basis of its LC-MS^2 spectrum (data not given) was proposed as the 2-(2-furyl)methylthio-1,4-dihydropyrazine (A in Figure 9). In addition, LC-MS^2 gave evidence that the ions at m/z=363 and 267 correspond to bis[2-(2-furyl)methylthio]-1,4-dihydropyrazine and 2-(2-furyl) methylthio-hydroxy-1,4-dihydropyrazine, respectively (A in Figure 9). To further confirm these structures, the experiment was repeated with [$^2H_2$]-labelled FFT (B in Figure 9). Comparing the LC/MS spectra (B in Figure 9) with those measured in the non-labelled experiment (A in Figure 9) revealed an isotopic shift of the ions at m/z 251 to m/z 253, thus confirming the incorporation of two deuterium atoms from the methylene group of one molecule of FFT as given in the structure proposed for 2-(2-furyl)methylthio-1,4-dihydropyrazine (*10*). In addition, an isotopic shift of two and four units were observed for the ions at m/z 267 to 269 and m/z 363 to 367, respectively, thus verifying the structures, outlined in Figure

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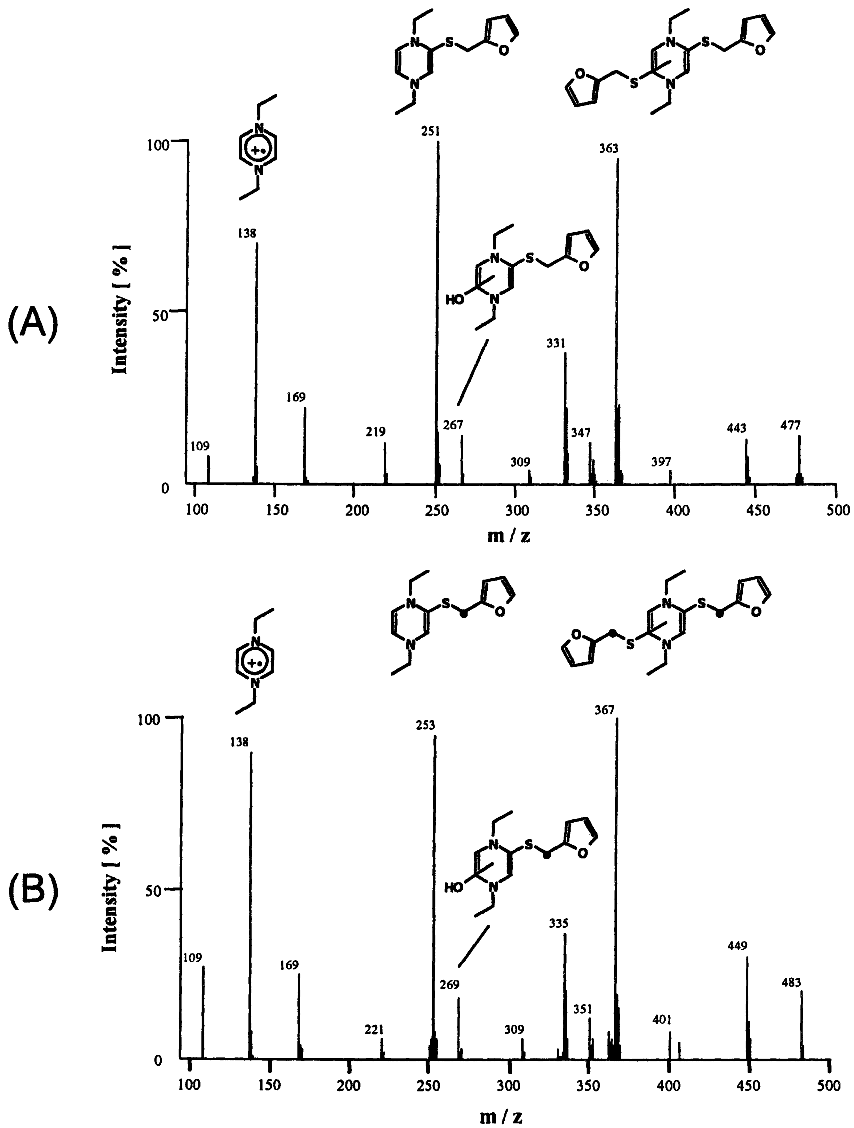

Figure 9. LC/MS spectra of aqueous solutions of Diquat and (A) FFT, and (B) $^{2}H_{2}$-FFT, respectively.

9, as 2-(2-furyl) methylthio-hydroxy-1,4-dihydropyrazine and bis[2-(2-furyl) methylthio]-1,4-dihydropyrazine (*10*).

To get more closer to the food, in an additional experiment, the CROSSPY radical was generated *in vitro* from N$_{\alpha}$-acetyl-L-lysine and glycolaldehyde prior to the addition of FFT. The aqueous solution containing CROSSPY and its corresponding pyrazinium-type redox partners (Figure 6) was incubated in the presence of FFT for 30 min at 30°C, and then analyzed by LC/MS spectroscopy

(*10*). The mass spectrum obtained showed a quasi molecular ion at m/z 537 being well in line with the structure of 2-(2-furyl)methylthio-1,4-bis-(5-acetamino-5-carboxy-1-pentyl)-1,4-dihydropyrazine, the structure of which is given in Figure 10. Fragmentation of that ion gave the MS^2 spectrum (ESI) displayed in Figure 10. Loss of 113 or 82, respectively, leads to the base ion at m/z 424 or 455, most likely corresponding to the cleavage of the 2-furfurylthio or the 2-furylmethyl group, respectively. The data clearly corroborated the results obtained for the Diquat model experiments and gave strong evidance that odor-active thiols are covalently linked to pyrazinium moieties in coffee melanodins.

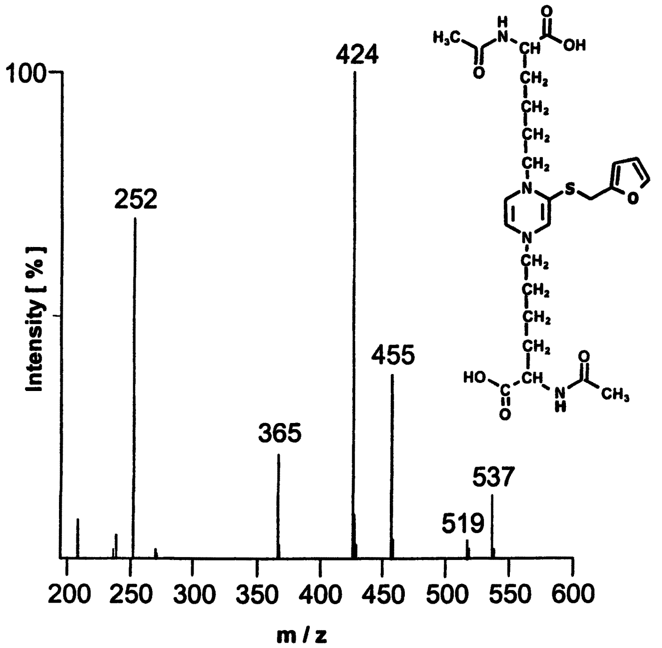

Figure 10. LC/MS^2 spectrum obtained for m/z 537 in a thermally pre-treated N$_\alpha$-acetyl-L-lysine/glycolaldehyde mixture after incubation (30 min/30°C) with FFT.

Taking all these data into account, the reaction pathways, displayed in Figure 11, were proposed for the binding of thiols to CROSSPY-associated reaction intermediates (*10*). Oxidation of CROSSPY (I) leads to diquaternary pyrazinium ions (II), which, in the absence of thiols, react with water to form the 2-hydroxy-1,4-dihydropyrazine III (*7,16,17*), or, in the presence of thiols such as, e.g. the 2-furfurylthiol, give rise to 2-(2-furyl)methylthio-1,4-dihydropyrazine (IV in Figure 11). Involving the diquaternary ions these intermediates participate in Redox reactions, thus pumping additional molecules of 2-furfurylthiol into the melanoidin via 2-(2-furyl)methylthio-hydroxy-1,4-dihydropyrazine (V) and bis[2-(2-furyl) methylthio]-1,4-dihydropyrazine (VI), respectively (*10*).

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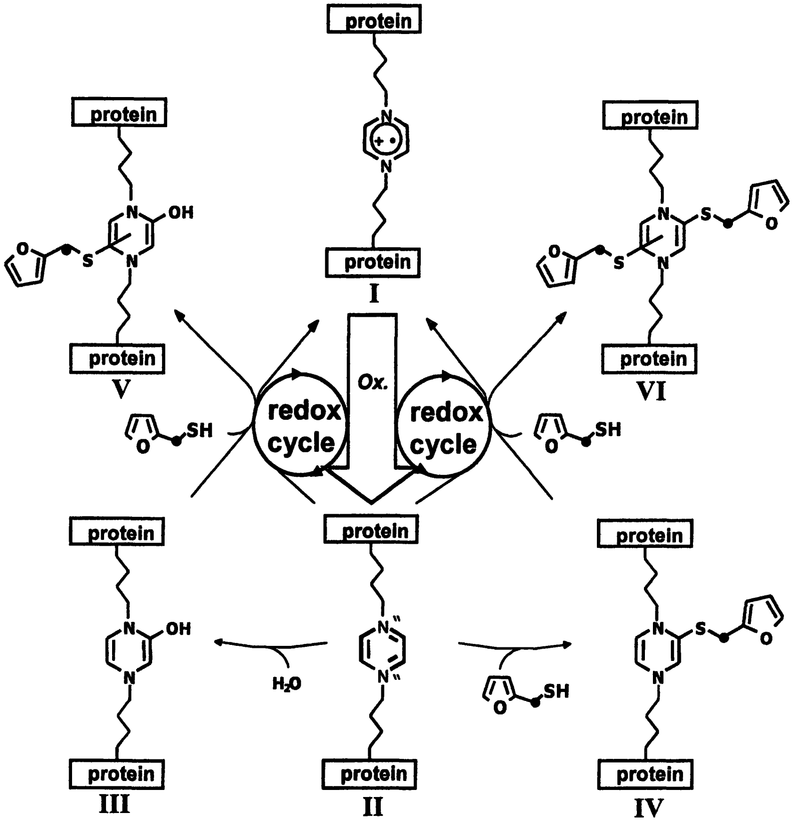
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Figure 11. Reaction pathways proposed for the covalent binding of 2-furfurylthiol to CROSSPY-associated reaction intermediates in melanoidins.

Thiol binding and radical activity in various food melanoidins

In order to investigate whether odor-active thiols bind exclusively to coffee melanoidins, or to melanoidins in browned foods in general, aqueous solutions of FFT were incubated for 30 min at 30°C in the presence of melanoidins isolated from bread crust, dark beer, roasted coffee, and dark malt, and the headspace concentrations of the "free" thiol were determined. As given in Figure 12, dark malt showed nearly identical thiol binding activity as roasted coffee, e.g. less than 10% of FFT were recovered only. In comparison, the melanoidins isolated from bread crust as well as dark beer showed somewhat lower reactivity towards thiol compounds, because about 55 to 60% of "free" thiol were determined after incubation. Analysis of these food melanoidins by means of EPR spectroscopy revealed that the radical activities run in parallel with the thiol binding activity, e.g. malt and coffee melanoidins showed the most pronounced effect in thiol binding and exhibited by far the highest radical activity, whereas bread crust and dark beer had the lowest potential in thiol binding and the lowest radical activity. These data again indicate the tight relationship between thiol binding and free radical activity of melanoidins, and clearly demonstrate that the binding of odorous thiols to melanoidins is not restricted to roasted coffee only, but is of general importance for the aroma of browned foods such as, e.g. cereals and beer.

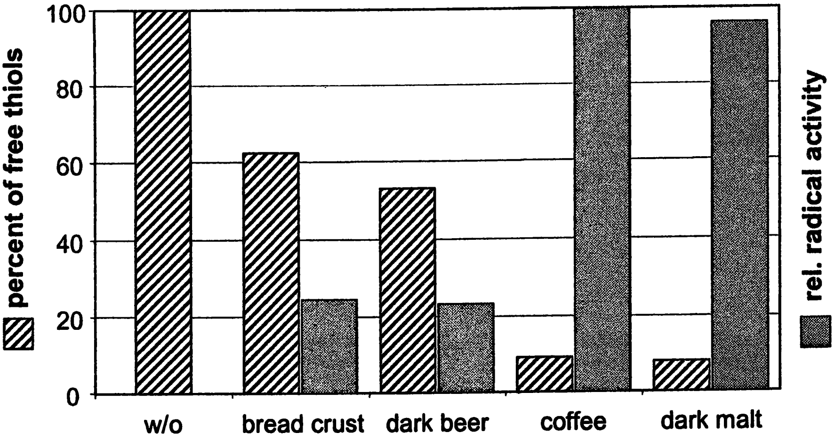

Figure 12. Amount of "free" FFT in the headspace of aqueous solutions of FFT incubated for 30 min at 30°C without (w/o), or in the presence of melanoidins isolated from bread crust, dark beer, coffee and dark malt.

Conclusions

On the basis of these results it might be concluded that CROSSPY-derived pyrazinium intermediates are involved in the rapid covalent binding of odorous thiols to coffee melanoidins, and are responsible for the decrease in the sulfury-roasty odor quality detected shortly after preparation of the coffee brew. Studies on how the activity of these binding sites might be influenced, e.g. by blocking the active binding sites, are ongoing and will help to elucidate novel possibilities how to stabilize the aroma quality and how to increase the aroma shelf-life of coffee-beverages.

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Chapter 16

Processing Green Tea Extracts to Make a Beverage Ingredient

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Aqueous extracts of green tea made from process quality green tea leaf possess many color and flavor related negative qualities that limit their use as a beverage ingredient. This paper discusses several approaches taken in processing green tea extracts to increase the acceptability of such extracts as beverage ingredients. They range from controlling the extraction conditions to ensure minimal post-extraction oxidation; removal of the already oxidized catechins by precipitation; and nanofiltration techniques which essentially clean up green tea extracts by removing the large molecular weight processing artifacts. Analytical data supporting the validity of such processing steps show that the essential character of green tea was retained through all of these clarification steps.

In Nutraceutical Beverages; Shahidi, F., et al.;
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Green tea is the preferred beverage of many East Asian countries and accounts for approximately 20% of the world tea market (*1*). All commercial tea, whether black, green, oolong or variations thereof are made from the tender leaves of *Camellia sinensis*. Of the two main varieties of *C. sinensis*, var. *assamica* with larger leaves is primarily used for manufacturing, of black tea while var. *sinensis* with much smaller leaves is used to manufacture green tea. Many crossbreeds of varying parentage exist in all of the tea growing countries.

Green tea leaf processing consists of the essential step of enzyme deactivation, which allows the polyphenols of the leaf to remain intact i.e. without oxidation. Leaves are subjected to steaming (Japanese method) or to panning (contacted with a hot metal surface – Chinese method) to deactivate the endogenous enzymes *(2)*. Quality green teas with good leaf attributes (intactness) are sold as commercial green teas for direct consumption while low quality broken leaf is sold primarily for further processing.

Good quality green tea extracts have a pale green color and a slight turbidity and are relatively mild tasting (ranging from brothy to leafy to slightly astringent) with a hay-like odor. Process (lower) quality green tea extracts are dark green/brown in color and taste very astringent and bitter. These taste characteristics are very different from the aroma and taste of black tea extracts, to which western consumers are accustomed and make green tea extracts difficult to formulate when compared to black tea extracts. The challenge for tea chemists are to make these green tea extracts taste more neutral (easier to formulate with) but yet have the essential tea character. This chapter describes various methods of clarification that are used to "clean-up" lower quality green tea extracts in order to provide an easily usable beverage ingredient.

Materials and Methods

Commercial samples of lower quality green tea were obtained through tea brokers listed in trade journals. None of the samples were sourced specifically from various countries or purchased from grocery stores.

HPLC analysis of green tea extracts

Caffeine and the four major green tea catechins namely EGC:(-)-epigallocatechin; EC:(-)-epicatechin; EGCG:(-)-epigallocatechin gallate; and ECG:(-)- epicatechin-gallate were analyzed using a reversed phase HPLC method. The HPLC analysis was performed with a Hewlett-Packard 1090 system equipped with a YMC basic column (250mm x 2 mm, 5 µm Waters, Milford, MA) and a Waters 486 variable wavelength detector set at 280 nm. To preserve the stability of the tea catechins under ambient conditions, a diluent consisting of 0.024% ethylenediamiretetrraacetic acid (EDTA), 0.1% sodium bisulfite and 10% acetonitrile in phosphate buffer (0.01M KH_2PO4, pH=3.1) was used to dilute the samples. Diluted samples were filtered through a Whatman Anotop 25 (0.2µm) inorganic membrane filter fitted to a 3ml disposable syringe.

The operating conditions of HPLC were as follows:
Mobile Phase: A= 0.01M potassium dihydrogen phosphate buffer, pH = 3.1, B= 10% water-90% acetonitrile. Flow rate was 0.4 mL/min with the following gradient: 0 min, 90% A + 10% B; 15 min, 80% A + 20% B; 18 min, 70% A + 30% B; 25 min, 70% A + 30% B (isoc. held); 30 min, 90% A + 10% B; and 35 min, 90% A + 10% B (isoc. held). A typical chromatogram is shown in Figure 1.

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Tea Screening

Chinese, Kenyan, Japanese and Sri Lankan green teas, Chinese oolong teas and commercial black teas were cold or hot extracted and the resulting extracts analyzed by HPLC for catechins and caffeine. For cold extractions time-temperature combinations of 16 h at 4°C or 3 h at 40°C followed by 15 min at 50°C were used. Hot extractions were carried out for 5-15 min at 90°C. Tea leaves:water ratios varied from 1:27.5 to 1:57. Tea extracts obtained by these experiments were analyzed for % soluble solids by refractometry and for catechins and caffeine by HPLC. Results are summarized in Table I.

Gelatin Fining

Chinese, Japanese and Sri Lankan green teas were cold extracted using the time temperature combination of 3 h at 40°C followed by 15 min at 50°C with a tea to water ratio of 1:27.5. Commercial gelatin (medium bloom, porcine) was dissolved in hot water (71°C) at a concentration of approximately 10% and added to the tea extract to obtain gelatin levels of 0.025, 0.05, 0.10 and 0.20% in the tea extract. Citric acid (0.25%) and sodium citrate (0.05%) with respect to the tea extract were also added to adjust the pH to about 3.5. The extracts were cooled to 10°C and centrifuged at 8,000 x g for 15 min. The supernatant was decanted from the tubes and the following measurements made. °Brix, absorbance at 600 nm, catechins by HPLC and astringency by sensory analysis using an expert panel. Results are given in Tables II and III.

Using Antioxidants and Acids in the Green Tea Extraction Step

Deionized water was heated to about 70°C and erythorbic, citric or phosphoric acids were dissolved to several different levels. Green tea leaves were then extracted in these solutions using the time-temperature combination of 3 h at 40°C followed by 15 min at 50°C with a tea water ratio of 1:27.5. The tea leaves were removed by crude filtration with two layers of muslin cloth and the resulting extract analyzed as before. The extract was fined with gelatin as reported in the previous experiment and the supernatant also analyzed for °Brix and catechins as described earlier. Levels of oxidized catechins were determined by the classical solvent extraction technique of Roberts and Smith (*3*) to obtain a relative value with respect to the treatments. Results are given in Table IV & V.

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Oxidative Stability of Various Tea Extracts

Green tea extracts, prepared in the following manner, were stored at about 60°C overnight in a water bath with access to air. Their absorbance at 430 nm was recorded over about 4 days.
Chinese green tea cold extracted in water
Chinese green tea cold extracted in water and fined with gelatin
Chinese green tea cold extracted in erythorbic acid solution and fined with gelatin
Chinese green tea cold extracted in erythorbic and citric acids solution and fined with gelatin. Results are given in Figure 2.

Nanofiltration Layout for Green Tea Extracts

Green tea extracts prepared by extracting green tea leaves in erythorbic and citric acid solutions were subjected to nanofiltration with membranes to remove the large molecular weight processing artefacts. The general layout is given in Figure 3.

Nanofilter Screening for Green Tea Extract Clean-up

Several nanofiltration membranes from Osmonics Inc, Minnetonka, MN, were screened to select the ideal membrane for extract processing. Results are given in Table VI.

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HPLC and Metal Ion Concentration Data for Green Tea Extracts Subject to Nanofiltration

Permeates and concentrates resulting from the two selected membranes were analyzed for their contents of catechins and caffeine by HPLC and calcium and magnesium by ICP (*4*). Results are given in Tables VII and VIII.

HPLC and Metal Ion Concentration Data for Green Tea Extracts Subject to Ion Exchange

Cold extracted green tea extracts were passed through an ion exchanger and subjected to HPLC (Table IX) and metal ion analysis (Table X).

Results and Discussion

The identity of the compound representing each peak (Figure 1) was confirmed based on the retention time and the UV-spectrum obtained from the photodiode array detecor and comparison with pure standards. Quantitation was obtained using separate calibration curves for caffeine and each of the four catechins.

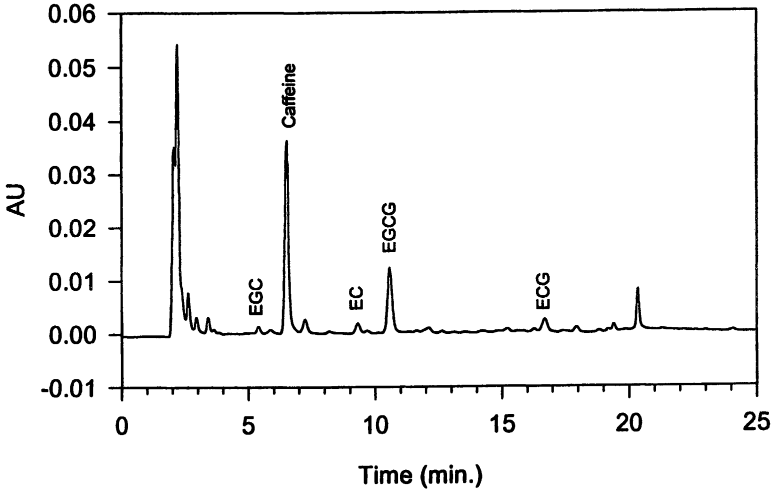

Figure 1. HPLC chromatogram of a typical green tea extract

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Some general trends were noted in the extracts made from various tea types as seen in Table I. Cold extractions carried out at 4°C in one stage yielded lower concentrations of all catechins compared to extractions done in two stages (footnote *a* samples vs. footnote *b* samples). Extractability of EGC anf EC were less dependant on temperature (footnote c samples), whereas EGCG and ECG were more extractable with increased extraction temperature (footnote d samples). Varietal differences also had an effect on the distribution of catechins as seen in the Japanese tea samples (primarily var. *sinensis*) which had a higher EGC+EC concentration vs. the Sri Lankan sample (var. *assamica*) which had a higher EGCG+ECG concentration. The partially oxidized oolong teas had lesser concentrations and the fully oxidized black teas had very small amounts of the monomeric catechins.

Table I. Type of tea, extraction condition, soluble solids, yield and concentration of caffeine and catechins

Tea type	Extraction conditions	% soluble solids	% yield	Concentration in extract (mg/L)				
				Caffeine	EGC	EC	EGCG	ECG
Kenyan	Cold[a]	0.67	18.19	278	740	273	437	103
Kenyan	Hot[b]	0.89	24.16	475	725	243	1067	239
Chinese-1	Cold[a]	0.74	20.09	393	538	177	430	132
Chinese-1	Hot[b]	0.81	21.99	606	411	135	925	232
Oolong	Cold[a]	0.79	21.44	268	594	150	357	65
Oolong	Hot[b]	0.63	17.1	575	436	200	634	134
Black	Cold[a]	0.81	21.99	246	0	0	25	0
Black	Hot[b]	1.07	29.04	672	0	0	162	0
Japanese	Cold[c]	0.91	Yields	798	1375	989	931	262
Japanese	Hot[d]	1.08	not	865	1300	926	1361	380
Sri Lankan	Cold[c]	1.01	recorded	1025	874	597	1674	440
Sri Lankan	Hot[d]	1.13	here.	1099	886	581	1957	526

[a] Tea leaves:water ratio 1:57; 16 h at 4.4°C

[b] Tea leaves:water 1:57; 5 min at 90°C

[c] Tea leaves:water ratio 1:27; 5. 1st 1:17.5, 40°C,2.5 hr then 1:10, 50°C,15 min

[d] Tea leaves:water ratio 1:27.5. 15 min at 90°C

Green teas of varying geographic origins subject to a range of extraction conditions yielded extracts with very much the same astringency, dark color and turbidity which forced us to look at other means of modifying the taste profiles of green tea extracts. Sensory astringency has been the subject of many investigations (*5*) and in general showed that larger molecular weight compounds provided a lingering astringency. Therefore, it was thought to apply this finding to cleaning up green tea extracts so they would be less colored and less astringent.

Table II. Effects of gelatin fining on green tea extract properties

Gelatin level (%)	Absorbance (600 nm)	% soluble solids	Astringency (Sensory)
0.00	0.068	0.65	+++
0.025	0.324	0.64	++
0.05	0.291	0.59	++
0.10	0.005	0.58	+
0.20	0.026	0.6	+

Table II shows the results of gelatin addition on sensory astringency. Not only did sensory astringency decrease substantially with increasing gelatin concentration, but the clarity of the extract (abs at 600 nm) also increased. However, there was a substantial reduction in the content of some of the catechins (Table III).

Table III. Effects of gelatin fining on the composition of green tea extracts

Gelatin	Concentration in extract (mg/L)				
level (%)	Caffeine	EGC	EC	EGCG	ECG
0.00	188	308	169	243	42
0.025	184	306	159	230	45
0.05	176	299	157	177	32
0.10	174	298	156	123	23

Note: Green tea extract was 0.4% soluble solids

This reduction was in accordance with the observations of Haslam (*6*) and Mehansho (*7*) where polyphenols of molecular weight > 500 had a higher affinity for gelatin like polymers rich in proline residues. Reductions in the concentration of both EGCG and ECG with molecular weights of 458D and 442D respectively are in keeping with this observation. As seen in Table III,

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both EGC and EC with lower MWs' were not affected by the gelatin fining step. Therefore by incorporating a gelatin fining step in the green tea extract process we could generate a milder green tea extract with much lesser formulation problems (*8*).

Oxidized polyphenols can be formed as a result of the extraction step where dissolved oxygen in water promoted further oxidation of reactive intermediates. To examine this problem erythorbic, citric and phosphoric acids were incorporated in the extraction step (Table IV). This also helped the formulators because these ingredients are commonly used in many beverages. The use of erythorbic acid as an antioixdant was reported by Esselen (*9*). To determine the effective use levels in these experiments, the effect of increasing concentrations on the reduction of absorbance at 430 nm (a good general indicator of colored oxidized polyphenols) was examined. Distinct effects appeared at a use level of about 500 mg/L. We added citric acid to aid in any possible metal chelation and also to reduce the pH to the high acid range to maintain process safety. Yield increases of up to 20% with respect to EGC, 30% with respect to EGCG and to over 40% with respect to ECG, occurred as seen in Table IV. Inexplicably, however, the yield of EC dropped by as much as 22%. Phosphoric acid did not help the yield increases broadly and in fact reduced EC yield by over 40%, while not influencing the increased EGCG and ECG yields. Gelatin fining did however exert strong binding effects on EGCG and ECG as

Table IV. Antioxidants use in green tea extraction – effect on catechins

Extract description	Concentration in extract (mg/L)				
	Caffeine	EGC	EC	EGCG	ECG
Chinese green tea - water extracted	1139	939	543	1573	276
Above extract after gelatin fining	926	763	448	836	156
Chinese green tea - acid solution extracted erythorbic (500 mg/L) + citric acid (0.2%)	1194	1129	556	2103	400
Above extract after gelatin fining	949	958	491	932	175
Chinese green tea - acid solution extracted erythorbic (750 mg/L) + citric acid (0.2%)	1230	1118	420	2156	386
Above extract after gelatin fining	1025	980	446	958	168
Chinese green tea - acid solution extracted erythorbic (750 mg/L) + phosphoric (0.2%)	1152	996	276	2027	367
Above extract after gelatin fining	920	902	376	903	154

Note: All extractions were 'cold' extractions as given in Table I, footnote *c*

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shown in Table III. Oxidized polyphenols in the gelatin fined extracts, were also reduced substantially as shown in Table V.

Table V. Antioxidants in green tea extraction – oxidized catechins

Tea type	Process condition	Oxidized catechins		Total color
		TF[a] (umol/L)	TR[b] (wt%)	
Chinese	Cold extracted	38.20	0.30	1.24
Chinese	Cold extracted & gelatin fined	21.10	0.25	1.08
Chinese	Cold extracted w/ erythorbic & citric acids & gelatin fined	5.51	0.19	0.45

[a]Theaflavins; [b]Thearubigins

Green tea extracts when stored with free access to air showed an almost linear increase in the absorbance at 430 nm(CGT) as shown in Figure 2. By gelatin fining of the extract, the tendency to oxidize is somewhat controlled and the absorbance curve shows no increase after about 24 h (CGT/G). By extracting the tea in an erythorbic acid solution and subjecting it to gelatin fining, more catechins are made available to oxidize over time and the absorbance curve showed a linear increase (CGT/E/G). Tea extracts made by extracting in erythorbic and citric acid solutions and subjected to gelatin fining, gave the best stability of all extracts (CGT/EC/G). It reached the same level of absorbance of the CGT/G curve but delayed the oxidation by over 60 h.

These experiments provided the necessary data to incorporate an antioxidant (erythorbic acid) and a metal chelating acid (citric acid) in the extracting medium to increase the oxidative stability of the green tea extracts (*10*). This improvement also rendered an unexpected increase in the yield of catechins from process quality green tea leaves. Thus, there were two important parts to processing lower quality green tea leaves to obtain a beverage compatible ingredient. The first of these was the use of reducing conditions to extract the solubles from tea leaves and the second was the removal of the high molecular weight fraction in the extracts. This second factor was accomplished by the use of a gelatin fining, a step commonly used in the juice processing industry (*11*).

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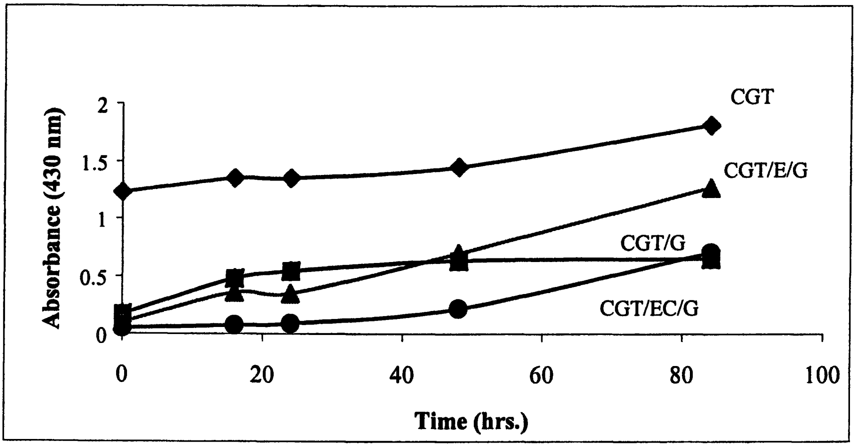

Figure 2. Oxidative stability of tea extracts under accelerated storage conditions.
CGT – Cold extracted chinese green tea. CGT/E/G – Cold extracted using erythorbic acid soution & gelatin fined. CGT/G – Cold extracted chinese green tea & gelatin fined. CGT/EC/G – Cold extracted using erythobic acid and citric acid solution & gelatin fined. Aging conditions are given in the Materials and Methods section.

Gelatin is an expensive material and in this particular use it binds irreversibly to the high molecular weight polyphenolics. Other means of removing high molecular weight compounds is to filter them from the solution. With this in mind, several membranes in the nanofiltration range to screen out the large molecular weight compounds were used. Nanofiltration (NF) experiments were typically done and permeates collected.

The green tea extract was kept warm (43-50°C) to minimize intermolecular interactions and pumped into a module housing the spiral wound NF membrane. The permeate (filtrate) was collected while the residue called the concentrate was recycled back into the feed. This experiment was used to first screen membranes that were compatible with green tea components and second to produce liter quantities of permeate for HPLC analysis to ascertain that indeed the right components in the permeate were attained.

When screening for compatible membranes, the flow rate of permeate and also the % soluble solids in the permeate as shown in Table VI were examined. Both the SP-12 and BQ-01 membranes gave good solids passage while the types

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Table VI. Membrane screening for green tea extract nanofiltration

Membrane Type	MWCO (D)	Composition based on	Permeate rate (L/h) 5 min	15 min	20 min	30 min	% solids Passage
SP-12	800	Cellulose acetate	28.9		26.1	25.8	>50
BQ-01	1000	Unknown	12.7			15.6	>50
SV-10	500		18.8		17.8		13
M-007	800	Unknown	18.8	18.4			16

SV-10 and M-007 gave very low solids passage. Permeate rates were low for all membranes except SP-12, which maintained a good flow rate throughout the experiment. The HPLC analysis of the permeates of both BQ-01 and SP-12 showed good catechins and caffeine passage as shown in Table VII.

Table VII. Green tea extract nanofiltration - Fraction compositions

Extract description	% soluble solids	Concentration in extract (mg/L) Caffeine	EGC	EC	EGCG	ECG
Unfiltered tea extract	2.00	1029	895	319	1668	320
BQ-01 membrane, permeate	0.74	435	390	157	367	73
SP-12 membrane, permeate: #1	0.72	472	437	207	588	111
SP-12 membrane, permeate: #2	0.74	483	464	206	595	121
SP-12, permeate (pooled)	0.82	602	550	244	733	151
SP-12, concentrate after 90 min.	4.05	1510	1428	499	2615	501
Gelatin fined unfiltered	1.85	854	831	301	787	162

As the filtration proceeded the components of interest were concentrated in the permeate as shown in the pooled permeate for the SP-12 membrane. When the catechins concentrations were standardized on the basis of 1°Brix, an enrichment of the catechins in the pooled permeates were observed. A corresponding decrease in concentration of catechins in the concentrate also occured. Table VII shows that compared to gelatin fining, membrane filtration is far more efficient at catechin recovery and as an added benefit, more cost effective in terms of the running costs.

An additional benefit of nanofiltration was the reduction in the concentration of calcium and magnesium ions. Table VIII shows that up to a 40% reduction in the Ca^{++} and Mg^{++} concentrations occured as a result of nanofiltration.

Table VIII. Green tea nanofiltration - metal ion reduction

Extract description	Concentration (mg/L / 1% soluble solids)	
	Calcium	Magnesium
Unfiltered tea extract	16.1	5.2
Gelatin fined	42.2	12.7
SP-12 permeate	10.4	3.0

To ensure other metal ion removal and provide greater stability to the extract a cation exchange step prior to the nanofiltration step in the green tea extract process was incorporated. This had no significant effect on the catechins or caffeine concentrations as shown in Table IX.

Table IX. Effect of ion exchange on green tea extracts

Extract description	Concentration (mg/L)				
	Caffeine	EGC	EC	EGCG	ECG
Feed	382	451	111	317	55
Ion exchanged	362	451	108	302	52

Table X. Effect of ion exchange on green tea extracts

Metal ion	Concentration (mg/kg)		%
	Feed	Ion exchanged	reduction
Aluminum	1323	625	52.8
Calcium	241	85	64.7
Iron	46	3	93.5
Magnesium	1770	5	99.7
Manganese	898	3	99.7
Potassium	20136	5050	74.9
Sodium	386	257	33.4
Zinc	27	1	96.3

Metal ions concentrations were reduced by up to 99% for magnesium and manganese. Zinc and iron concentrations were reduced by upto 96% while the least affected was sodium (Table X). The tea plant tended to accumulate aluminum and it held back in the extract also. Potassium levls were also high in

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tea extracts as previously reported (*2*). The flow rates through the ion exchange column can be optimized for greater metal ion recovery, however our interest was to reduce primarily the transition metal ions such as iron and manganese which have been implicated in the oxidation of catechins (*12*).

Improvements to green tea extract processing by including extraction of the leaves using antioxidants, passing the extract through a cation exchanger and subjecting the extract to nanofiltration to remove the high molecular weight oxidized materials are covered in several US Patents (*13,14,15*).

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Chapter 17

The Influence of Roasting-Derived Polymeric Substances on the Bitter Taste of Coffee Brew

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The isolation, chemical characterization and taste properties of bitter-tasting fractions of decaffeinated coffee brew is described. The flavor of brewed coffee consists of an alluring aroma coupled with a pleasant bitter/astringent taste making the foodstuff highly acceptable to consumers worldwide. In a given population the appeal of coffee flavor depends on an optimum level of bitterness. The bitter taste of coffee brew has been attributed to natural products in raw beans and novel compounds formed during roasting. Chemical and spectroscopic evidence suggest that chlorogenic acids and their roasting products may be a cause of the observed bitter taste. The bitterness of brewed coffee was reduced by admixing solvent-defatted spent coffee grounds with ordinary grounds prior to brewing.

In Nutraceutical Beverages; Shahidi, F., et al.;
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Because the flavor of coffee beverages is so widely accepted worldwide the chemical factors underlying the flavor are of great interest and have been thoroughly studied. Many aspects of coffee flavor are important for its acceptance including aroma, taste, texture and appearance, i.e., color. Coffee research has focussed mainly on aroma and modern analytical techniques have led to the identification of over 800 volatile compounds. In spite of such apparent chemical complexity careful organoleptic studies have recently shown that coffee aroma can be artificially reproduced by a mixture of only twenty-seven key odorants (*1*).

Less attention has been devoted to the non-volatile components of coffee flavor and the chemistry of coffee taste is only partially understood. Coffee brew contains about one percent dissolved solids that give rise to its sour, metallic, bitter and astringent taste impressions. The sour/metallic (salty) aspect of taste has been reasonably explained by the presence of numerous organic and mineral acids and their metallic salts (*2*). Coffee brew is in fact a complex buffer system whose sour/metallic taste ratio can be dramatically affected by small changes in pH (2). Superimposed on the sour/metallic taste are the effects of bitterness and astringency. The causes of bitterness perception in coffee have already been addressed by several researchers (*3*) and they were not pursued by us *per se*. Rather, it was our intent to devise a practical means for selectively controlling the bitterness component of coffee flavor during brewing. Toward this end we isolated a bitter fraction from coffee and used it as a model in adsorptive studies aimed at reducing bitterness. Adsorptive technology had previously been reported to benefit coffee flavor, but several shortcomings were apparent in efforts published prior to our study (*4*).

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Experimental

Isolation of Bitter-tasting Fractions From Coffee

A cylindrical plastic vessel equipped with a drain plug and a coarse filter was charged with one pound of commercial (drip grind) decaffeinated roasted and ground (RG) coffee and 2.0 L of distilled water. After 16 h at 22°C the mixture was drained by gravity and the retained grounds were washed with fresh water. The damp grounds were re-brewed in ca. 100 g portions in a standard-sized (12 cup) Proctor Silex coffee maker equipped with conical paper filters. Each 100 g portion was extracted with one pot of water and the volume of the combined extractions was reduced to ca. 500 mL by vacuum rotary evaporation. Finally the product was frozen and freeze-dried to obtain ca. 17 g of a solid, ca. 4% of the original RG weight. The product was dialyzed by conventional static dialysis using cylindrical (32 mm d) cellulose tubing (SpectraPor No. 1, MW cutoff 6-8 kDa). Typically 1.4 g of re-brew solids suspended in 50 mL of water was dialyzed against water at 22°C for 3 h vs. 2 L and 16 h vs. 2L to obtain after vacuum concentration and freeze-drying 0.69 g of dialyzed re-brew solids (DRS) of MW < 8 kDa and 0.66 g of undialyzed re-brew solids (URS) of MW > 8 kDa. The bitter-taste properties of the dialysis fractions were assessed by tasting serial dilutions of the materials in water (Table I).

Table I. Taste Properties of Coffee Extracts

Material	% in Water	*Taste*
DRS (MW < 8 kDa)	0.1	Extremely bitter
DRS (MW < 8 kDa)	0.05	Bitter
DRS (MW < 8 kDa)	0.01	Trace bitter
URS (MW > 8 kDa)	0.1	Gritty, ashy and smoky
5-CQA (K-salt)	0.1	Tasteless
Quinine-HCl	0.001	Bitter

DRS, dialyzed re-brew solids; and URS, undialyzed re-brew solids.

Table II. Elemental Composition of Coffee Fractions (%)

Material	C	H	N	O	Ash
DRS (MW < 8 kDa)	49.05	5.00	1.60	31.63	12.72
URS (MW > 8 kDa)	51.49	6.09	3.39	36.69	2.90
5-CQA (K-salt)	48.9	4.33	0	36.7	10.0
	K	*Na*	*Ca*	*Mg*	*Total*
DRS (MW < 8 kDa)	11.7	0.191	0.044	0.033	11.97
DRS (MW > 8 kDa)	0.89	0.069	0.31	0.224	1.49
5-CQA (K-salt)	10.0				10.0

Percent oxygen determined by difference

Analysis of DRS and URS Fractions

Combustion analysis showed that C, H, K, N and probably O were major elements in both dialysis products (Table II). UV spectra were obtained in dilute aqueous solution and FT-IR spectra were obtained on solid samples using

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standard attentuated total reflectance (ATR) methodology. The more bitter-tasting DRS fraction was further analyzed by ion-exchange (IE) and high performance liquid chromatography (HPLC) (Table III).

Table III. Ion-exchange and HPLC of Coffee Extract DRS

Fraction →	*Initial DRS*	*Neutral acid*	*Basic*	*Pyridine*
Relative Amount →	---	*75%*	*0%*	*14%*
Taste @ 0.05% →	*bitter*	*bitter*	*none*	*sl. Bitter*
HPLC Data	%	%		
3-CQA	4.00	3.08	--	--
4-CQA	5.16	3.56	--	--
5-CQA	8.47	6.26	--	--
3-FQA	0.52	2.55	--	--
5-FQA	1.18	1.05	--	--
3,4-diCQA	2.34	0.14	--	--
3,5-diCQA	1.75	NF	--	--
4,5-diCQA	2.65	NF	--	--
PPA-1	0.47	0.28	--	--
PPA-2	NF	0.09	--	--
PPA-4	0.47	NF	--	--
PPA-5	4.18	0.34	--	--
PPA-6	3.38	0.59	--	--
Total identified	34.6	17.9	--	--

CQA = caffeoylquinic acid, FQA = feruoylquinic acid, PPA = polyphenolic acid of unknown structure. Tasting done after pH was adjusted to 5.4 with KOH. NF = none found

For ion-exchange separation an aqueous solution containing DRS was allowed to flow through a column of sulfonated polystyrene resin (100 mesh, H+ form) and vacuum evaporated to obtain a neutral + acidic fraction. Further elution with 0.1 N ammonium hydroxide and pyridine yielded fractions containing basic and strongly adsorbed organic materials, respectively. Some fractions were analyzed by HPLC using previously published procedures (*5*).

Adsorbant Screening Method

Aqueous solutions containing 1.0 mg/mL of DRS (25 mL) were treated with 1, 10 or 100 mg of insoluble adsorbant candidates and stirred magnetically at

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22°C. At time intervals samples were withdrawn and analyzed by UV (320 nm) to assess residual DRS content. For quantitation a calibration curve was prepared with DRS and used with LOTUS 123 software to produce time-dependant adsorption data in graphical form. Representative data points were derived from the graphs to make practical comparisons between various adsorbants (Table IV).

Table IV. Adsorption of DRS by Solid Adsorbants

Solid Adsorbant	*% DRS Removed From 0.1% DRS Solution in 2 h @ 22°C*
Activated charcoal	99
Alumina	66
Hydroxyapatite	15
β-Cyclodextrin polymer	50
Chitosan	28
Alginic acid	23
Polyvinylpyrrolidone (cross-linked)	39
Nylon-6	29
Lactalbumin	32
Corn zein	20
Keratin	19
Defatted spent coffee grounds	14

Initial adsorbant to substrate weight ratio = 10:1

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Preparation of Defatted Spent Coffee Grounds (DFG)

A detailed description of DFG preparation and use is published elsewhere (*4*). In brief, damp extracted coffee grounds were stirred at 50-60°C in a mixture of ethanol, ethyl acetate and water, filtered and desolventized under vacuum to obtain a fine brown powder.

Results and Discussion

Isolation and Characterization of a Bitter-tasting Fraction From Coffee Brew

A bitter-tasting fraction was isolated from decaffeinated RG coffee by a two step extraction procedure. In the first step the coffee was extracted with water at room temperature (ca. 22°C) for 16 h to remove mainly non-bitter solubles.

Typically, 15% of the coffee is solubilized under these conditions. The damp grounds from room temperature brewing were reextracted with hot water (ca. 85°C) to afford a bitter-tasting fraction designated as rebrew solids (RS). After freeze-drying the RS was obtained as a fluffy grey matter amounting to ca. 5% of original RG weight. A 5% aqueous solution of RS was colored black and had a pronounced bitter-dirty-ashy taste. Equilibrium dialysis of RS separated a bitter-tasting fraction (< 8 kDa) designated dialyzable RS (DRS) from a more burnt-ashy tasting part (> 8 kDa) termed undialyzable RS (URS). Taste evaluations (Table I) indicated a bitter taste threshhold for DRS at ca. 0.01% in water or approximately one-tenth the bitterness of quinine hydrochloride. The more bitter-tasting DRS material was subjected to further study including proximate chemical analyses.

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Combustion analysis of DRS (Table II) indicated a carbohydrate-like material similar in composition to the chlorogenic acids well known to occur in coffee (*2*). The high ash content (mainly potassium) is explained if acidic materials, i.e., phenolic acids are partially present as metallic salts. The low level of nitrogen (1.6%) and a weak ninhydrin test indicated the possibility of a small amount of soluble proteins or Maillard reaction products.

Aqueous solutions of DRS absorbed strongly in the ultraviolet producing a spectrum closely resembling the spectrum of 5-caffeoylquinic acid (5-CQA) with a UV max at 320 nm. However, the absorbance of DRS at its UV max was only 50% that of pure 5-CQA. Consistent with phenol chemistry the peak at 320 nm disappeared in 0.1 N sodium hydroxide solution giving rise to a weaker phenolate ion absorption centered at 370 nm. The IR spectrum of DRS qualitatively resembled the IR spectrum of 5-CQA, however, a reversal in absorption intensities at 1600 and 1700 cm^{-1} was observed that was probably due to partial salt formation in the coffee isolate. Also, DRS has unique bands at 1780 and 760 cm^{-1} which are not found in the spectrum of 5-CQA.

The DRS was fractionated into neutral-acidic and basic components via an ion-exchange procedure in order to locate the chemical origin of its bitter taste (Table III). Neutral-acidic material comprised 75% of the DRS and retained most of the bitter taste. Continued elution of the resin with dilute ammonia solution and pyridine gave 14% of basic and/or highly adsorbed materials which had less bitter taste and were not investigated further. The balance of the DRS weight (ca. 11%) was presumed due to metallic ions which were not eluted from the resin.

HPLC analysis of DRS identified fourteen phenolic acids comprising 35% of the sample weight. Of the fourteen compounds eight were identified as mono or dicinnamoylquinic acids based on comparison with reference standards and six were tentatively identified as polyphenolic acids (PPAs) and assigned phenolic acid status based solely on UV spectral data. Similar analysis of the bitter-tasting neutral-acidic IE fraction indicated that it contained less total phenolic acids compared with the parent DRS (18% vs 35%) and that the difference resulted from less dicinnamoylquinic acids and PPAs in the IE fraction. Since DRS and the derived neutral-acidic IE fraction had comparable bitter taste at 0.05% it seemed unlikely therefore that the dicinnamoylquinic acids or the PPAs are the cause of the bitter taste. In spite of this result phenolic acids (of undetermined structure) may yet be responsible for bitter taste since total phenolics in DRS assessed by UV absorption (50%) minus 35% identified by HPLC still leaves 15% unidentified phenolic acid materials with unknown organoleptic properties.

Use of Solid Adsorbants to Reduce the Bitter Taste of Brewed Coffee

Based on the foregoing experiments we hypothesized that polyphenolic compounds (of unidentified structure) might be responsible for the excessive bitter taste in brewed coffee and that these materials could be reduced or removed by adding an insoluble adsorptive material to RG coffee prior to brewing. To expedite the selection of adsorptive materials we devised a screening procedure in which adsorbants were exposed to dilute aqueous solutions of DRS. The loss of DRS from solution was interpreted as a measure of adsorbant effectiveness (Table IV). The effectiveness of proteinacious materials was of special interest to us in view of safety concerns surrounding all new food additives. It occurred to us that spent (pre-extracted) coffee grounds might also function as an adsorbant since they are known to contain about 14% protein. Spent coffee grounds are a common by-product of instant coffee manufacturing with little current value apart from use as a fuel or as landfill. As described previously (*4*) spent grounds are solvent-extracted and dried to remove excess lipids and water and pulverized to obtain a finely divided, odorless, tasteless adsorbant material. The defatted grounds (DFG) exhibited relatively weak adsorbancy (14%) in our DRS screening assay at 22°C, but the material showed promise for flavor modification in a practical test in which 5.7% DFG was added to RG coffee prior to brewing (Table V). Addition of DFG to RG coffee under conventional brewing conditions produced a brew that was

Table V. Flavor Effects of Defatted Coffee Grounds (DFG) in Brewed Coffee

	Averaged panelists' scores	
Flavor attribute	*Control*	*Control + 5.7% DFG*
Aroma (overall)	28.1	26.3
Burnt aroma	12.3	6.5
Taste intensity (overall)	33.3	30.6
Bitter taste	26.8	21.1
Burnt taste	21.0	15.9
Ashy taste	16.0	13.1
Color	32.5	32.3

Scale = 0-60 panel units; number of panelists = 11

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perceived by expert taste panelists to have significantly reduced bitter taste, burnt taste and burnt aroma. The actual cause of bitterness perception in brewed coffee was not determined in this work and it remains as an area for future research.

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Chapter 18

The Role of Flavanols and Their Related Oligomers in Cardiovascular Health

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Cardiovascular disease is a leading cause of death in most developed countries. Numerous epidemiological studies have provided evidence for the concept that the chronic consumption of flavonoid-rich diets can be associated with a decreased risk for coronary heart disease and stroke. In addition to select fruits, vegetables, nuts and beverages such as red wine and green tea, certain chocolates have been shown to be rich in flavonoids, particularly the flavanol (-)-epicatechin and its related oligomers. Several investigators have reported that these compounds can have a number of important physical and biological properties. For example, it has been shown *in vitro* that flavanols and their related oligomers can act as potent antioxidants, reduce platelet activation, promote endothelium-dependent vasorelaxation, and modulate eicosanoid production. Significantly, biological effects observed with the flavanols are often quite different than those observed with the oligomers; thus there is considerable structure specificity. While the potent *in vitro* effects of the flavanols and their related oligomers have generated considerable interest in these compounds, more data concerning their *in vivo* effects would be beneficial. Pilot clinical studies with tea, red wine, and chocolate will be reviewed during this presentation. The data support the concept that foods rich in flavonoids, and particularly in flavanols and their related oligomers, can contribute to a heart healthy diet.

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As a consequence of their ability to influence several parameters associated with cardiovascular health, dietary flavonoids, and specifically the flavanols and their related procyanidin oligomers, have become the focus of considerable attention. As is reviewed below, the concept that certain flavonoids may provide beneficial vascular effects is supported by observations on their ability to act as antioxidants (e.g., they can inhibit the rate of LDL- oxidation), as well as their ability to decrease platelet aggregation, modulate eicosanoid production, and enhance vascular endothelial function. Epidemiological evidence, demonstrated by many large observational studies, has generally supported a beneficial effect of flavonoids on cardiovascular health. For example, as dietary flavonoid intake increased from 0-19 mg/d to > 29 mg/d among a cohort of men participating in the Zutphen Elderly Study (*1*), the risk of mortality from coronary heart disease decreased by 60%. However, the flavonoid-rich foods accounted for in the diets of those in the Zutphen Study included black tea, onions, and apples, due largely to the narrow compositional definition; i.e., only five flavonoid compounds were analyzed in specific foods in the diet. Thus, the validity of these results is questionable. Furthermore, Rimm *et al.* (*2*) found little if any benefit associated with flavonoid consumption using the same narrow compositional definition as Hertog *et al.* (*1*).

The extent to which different flavonoid-rich foods may contribute to cardiovascular health is unknown. However, Arts *et al.* (*3*) recently suggested that the intake of flavanols (~ 72 mg/d), one type of flavonoid, could partially explain the inverse relationship between tea consumption and ischemic heart disease in these men. While the epidemiological data generally provide support for the concept that the flavonoid content of plant- based foods is critical to their influence on cardiovascular disease risk, these observations are associative at best, and provide limited information on the differential effects of various flavonoids. If the bioavailability, and more importantly, the bioactivity of various flavonoids are markedly different, this may have significant implications for dietary recommendations. The report herein will focus on these aspects of flavonoids, with particular focus on flavanols and flavanol-related procyanidin oligomers, and their influence on cardiovascular health following the dietary consumption of flavonoid- rich beverages such as red wine, green and black tea, purple grape juice, and cocoa.

Antioxidant Activity

A growing body of research suggests that excessive tissue and cellular oxidative damage may play a significant role in cardiovascular disease. Likewise, it has been hypothesized that the dietary intake of antioxidants may modulate the risk for cardiovascular disease. It is generally accepted that flavanols and their related procyanidin oligomers are antioxidants by virtue of their chemical structures (Figure 1), and considerable research has focused on whether their antioxidant properties confer protective effects on cardiovascular health. For example, a lower than expected incidence of heart disease in France has been attributed in part to a relatively high red wine consumption in their population (*4*). Frankel and co-workers (*5*) suggest that the putative protective effects of red wine could be attributed in part to the antioxidant properties of the polyphenols in the beverage. Consistent with this, Whitehead and colleagues (*6*) subsequently demonstrated that following the consumption of 300 mL of red wine, serum antioxidant capacity was significantly increased in healthy adult subjects and that the effects seen were similar to those observed following supplementation with 1000 mg of ascorbic acid. However, these antioxidant effects were not seen following the consumption of white wine. One explanation for the differences between red and white wine could have been attributed to the greater presence of flavonoids, including flavanols and procyanidins, in red wine as compared to white wine.

Flavanols and their related procyanidin oligomers can act as antioxidants by several mechanisms, including hydrogen donation, quenching singlet oxygen, scavenging radicals, and chelating redox active metals (*7-9*). The antioxidant properties of select flavanols and procyanidin oligomers have been demonstrated in foodstuffs (*10*), synthetic liposomes (*11*), ex vivo human plasma (*12*), and animal models (*13*). Adamson and co-workers (*14*) demonstrated a strong relationship between the flavanol and procyanidin content in cocoa and chocolate and their antioxidant capacities as measured using the oxygen radical absorbance capacity (ORAC) assay. To test the concept that increased serum antioxidant capacity observed following consumption of a flavanol-rich food is due to the flavanols, Wang and co-workers (*14*) conducted a clinical study in which subjects consumed increasing amounts of chocolate (0, 27, 53, and 80 g) containing 6.87 mg of flavanols and procyanidins per gram of chocolate. Following consumption of chocolate, a dose response relationship (Figure 2) was observed between the levels of epicatechin (one flavanol present in chocolate) found in the plasma and a trend for increased serum antioxidant capacity and decreased lipid oxidation products. It was suggested that the positive changes observed on oxidative status could be due in part to the epicatechin circulating in the blood.

The antioxidant activity of flavanols and their related oligomers observed in flavonoid- rich beverages and foods is further exemplified by effects observed on LDL oxidation, a biomarker believed to play a role in cardiovascular disease (*15*).

Inhibition of LDL oxidation

Flavonoids, including flavanols and procyanidins, have been extensively investigated for their ability to inhibit LDL- oxidation, in both *in vitro* systems as well as *in vivo* supplementation studies of flavonoid- rich foods and beverages. The oxidative modification of LDL is widely recognized to play a pivotal role in the formation of foam cells and subsequent development of atherosclerosis. LDL oxidation can take place within the arterial wall and under other conditions of oxidative stress, while macrophages and other cells, including endothelial cells and smooth muscle cells, can also oxidize LDL. The

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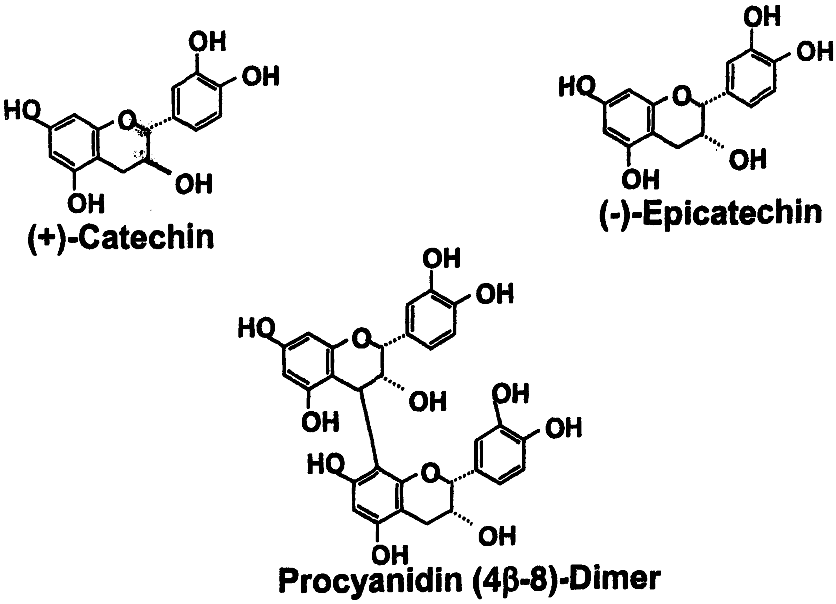

Figure 1. Chemical structures of flavonols.

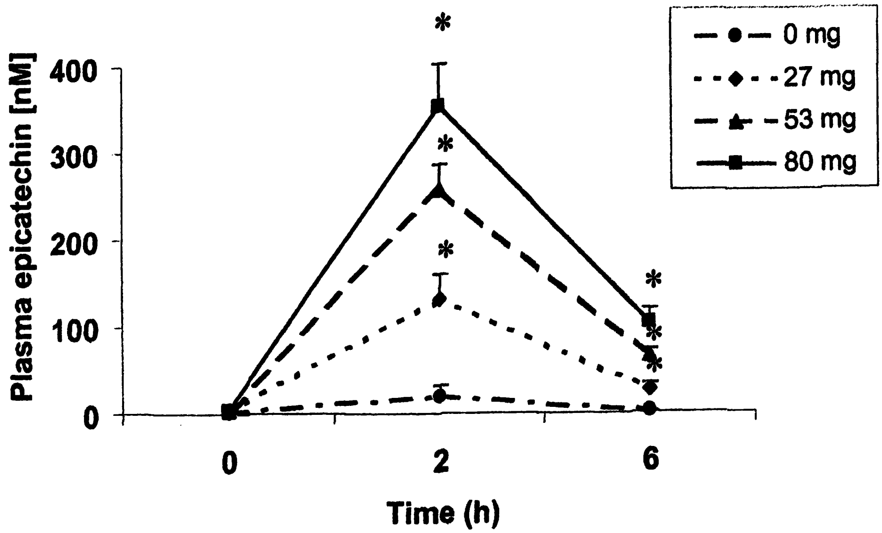

Figure 2. Dose-response effect of plasma epicatechin following consumption of flavonoid-rich chocolate (ref. 14).
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susceptibility of LDL to oxidation may therefore serve as a biomarker for the presence, severity, and progression of the disease. Not surprisingly, researchers have focused on the potential role of certain flavonoids to prevent LDL oxidation, thereby preventing or slowing the atherosclerotic process. Powerful antioxidant activity of flavonoids may be responsible for this effect, since they have been shown to scavenge free radicals, chelate redox active metals, donate hydrogen, and spare vitamin E, all of which are antioxidant activities that may lead to effectively inhibiting LDL oxidation (*16*).

Several flavonoids, including flavanols and procyanidins, and flavonoid-rich foods have been studied for their potential to inhibit LDL oxidation. It has been previously been reported that the phenolic substances found in grapes and red wine, such as flavanols and their related oligomers, the procyanidins, inhibit LDL oxidation in vitro (*5*). A variety of flavonoids, like those found in chocolate, the flavan-3-ols, epicatechin and catechin, and their related oligomeric procyanidins, have also been shown to protect LDL from oxidation after their addition to *in vitro* oxidation systems (*17*). Supplementation studies with red wine, purple grape juice, and cocoa have also shown beneficial effects in preventing LDL oxidation, while studies investigating the effects of green and black tea have been equivocal.

A recent clinical intervention involving the daily consumption of a cocoa beverage investigated the effects of cocoa- derived flavonoids on the susceptibility of LDL to oxidation (*18*). In this study, subjects consumed 36 g of cocoa, which provided 2610 mg of total polyphenols (measured using a non-specific assay) daily for 2 weeks. The primary polyphenols in cocoa are the flavanols and their related procyanidin oligomers, with minor amounts of other flavonoids. A control group consumed an equivalent amount of sugar as contained in the cocoa beverage. Using either V-70 (2-2'-azobis 4-methoxy-2,4-dimethylvaleronitrile) or $CuCl_2$ to initiate radical formation, susceptibility of LDL to oxidation was determined in LDL isolated from the plasma. Compared to baseline, lag time to oxidation of LDL was significantly increased in the cocoa group by 29% using the V-70 radical initiator, and by 14% using the Cu ion initiator (Figure 3). It is important to note that the authors of this study reported no change in plasma vitamins E, C, and β-carotene after a 2-week cocoa beverage intervention. When blood samples were drawn, the investigators were unable to detect the primary flavanol (epicatechin) in the plasma, although an increased urinary excretion of epicatechin was observed 1 and 2 weeks after cocoa consumption. It is possible that the timing of blood draws had a significant effect in the detection of cocoa flavanols in plasma since blood samples were taken following a 12-hour fast. The inability to detect the cocoa flavanols was most likely due to the fact that these compounds are excreted within 8 h (*14,19,20*). Given that there was a significant increase in lag time to LDL oxidation after 1 and 2 weeks of cocoa consumption, and that no changes were found in plasma antioxidant vitamins, suggests that the protective

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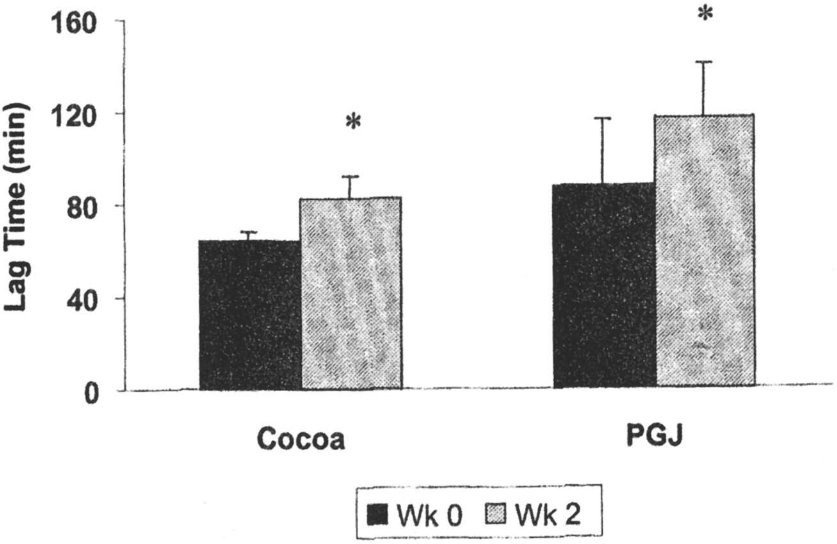

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Figure 3. Inhibition of LDL oxidation with flavonoid-rich beverages. (18, 22.)

effect on LDL oxidation may have been directly due to the antioxidant activities provided by the cocoa flavanols. Alternatively, chronic consumption might be associated with an increase in the amount of vitamin E, or other antioxidants, associated with the LDL particle. However, in general flavanols are assessed in the plasma, and are rarely assessed in LDL particles that are isolated from plasma. Regardless of the mechanisms involved, these results provide additional evidence for cocoa- derived flavanols to prevent LDL oxidation, and build upon results reported in a previous study by Kondo *et al.* (*21*).

In this earlier investigation, the susceptibility of LDL to oxidation was determined following the consumption of 35 g of defatted cocoa by human volunteers. LDL that was isolated from plasma, 2 h following cocoa consumption, exhibited a lag time to LDL oxidation that was significantly increased by 15%. Taken together, these studies demonstrate that consumption of a flavonoid-rich cocoa product can be associated with inhibition of LDL oxidation under both acute (2 h) and chronic (14 days) conditions.

The daily consumption of purple grape juice has also been investigated for its potential effects on susceptibility of LDL to oxidation, as well as for its effects on endothelial function (*22*). Patients with coronary artery disease consumed ~ 21 oz. of purple grape juice daily for 2 weeks. In addition to improving endothelial function (discussed under the section *Vascular Endothelial Function*), lag time to oxidation of LDL in the formation of conjugated dienes significantly increased by 34.5% (Figure 3). Although plasma flavonoid concentrations were not assessed in the subjects consuming the juice, it is reasonable to speculate that certain flavonoids in grape juice may be responsible for the effects seen *in vivo* in this study.

The consumption of flavonoid- rich beverages to prevent LDL oxidation has not always produced consistent results. For example, Hodgson *et al.* (*23*) investigated the acute effects of black and green tea consumption on LDL oxidation, and found that although consumption of tea beverages led to measurable increases in urinary 4-O-methylgallic acid (a marker of uptake and metabolism of polyphenols from tea), there was only a mild increase in lag time to LDL oxidation from the consumption of black tea, and a trend for an increase after green tea consumption. Similarly, van het Hof *et al.* (*24*) investigated the consumption of green or black tea on LDL oxidation in subjects who were instructed to consume one cup of tea every 2 h for a total of eight cups per day for three days. Although tea flavanols were found to be bioavailable from both green and black tea, when the susceptibility of LDL to oxidation was measured using Cu- induced oxidation at 60 h as compared to baseline, investigators found that the tea flavanols were unable to protect LDL from oxidation. One explanation for this lack of an effect of tea consumption on LDL oxidation, as offered by the authors, may have been due to the timing of the blood draw. Since the half- life of plasma flavanols is only 2-4 h, perhaps their fasted blood contained only minor amounts of tea flavanols. This would imply, however,

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that protection of LDL from oxidation is an immediate effect, or at least relatively acute (3 days or less) versus chronic (> 2 weeks). However, as discussed previously, protection may be through secondary effects such as sparing of vitamin E in the LDL particle, as demonstrated by Lotito and Fraga (*25*), which would require a longer time to observe. The lack of effect observed in these tea studies conflicts with the purple grape juice and cocoa studies that were discussed earlier in this section, and other tea research previously conducted. An additional explanation for results from these tea interventions could be that the consumption of these specific flavanol- rich tea beverages (processing, preparation, etc.) required a longer time for an effect to develop, such as those seen with the cocoa and grape juice beverages.

Although much attention has been given to the antioxidant activity of flavanols and procyanidins as a potential mechanism for their protective action, the biological implications of such measures are not yet clear and the role of oxidative damage as a primary cause of cardiovascular disease has been challenged by some researchers. Thus, it is important to consider that other mechanisms in addition to antioxidant activity are likely to be involved in their apparent cardioprotective effects.

Platelet Aggregation

Frequently, the first clinical manifestation, and often a fatal event in cardiovascular disease, is platelet adherence to and aggregation on the atherosclerotic vessel wall, resulting in thrombosis. Preventive antithrombotic treatments include platelet inhibitors such as aspirin. It has recently been suggested that flavanols and their related oligomers may affect thrombosis, and in doing so, affect the risk for cardiovascular disease, by interfering with platelet activation and function.

Blood platelets circulate in the bloodstream as small, anucleate disk-shaped cells and are critical for the maintenance of the integrity of the vascular system. Under normal conditions, the endothelium releases platelet-inhibitory modulators such as nitric oxide (NO) and prostacyclin. Upon vessel wall injury, factors are exposed at the site of vascular disruption, which allow platelets to recognize and adhere to the site of injury, change shape, secrete contents of various intracellular granules, and finally, aggregate to form a thrombus. The state of platelet activation is dynamically controlled through a diverse array of stimulatory and inhibitory signals to which the platelet may be exposed. The platelet surface has specific plasma membrane receptors that can recognize these signals and occupancy of platelet receptors can initiate a series of biochemical changes mediated by intracellular second messengers.

It has been demonstrated that flavanols and their related oligomers can inhibit platelet activation *in vitro*, following stimulation with epinephrine (*26*). Importantly, these effects have also been observed following the consumption of

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flavanol-rich beverages such as cocoa, tea, and purple grape juice. Using flow cytometry, and monoclonal and secondary fluorescent antibodies, Rein and co-workers (*26,27*) measured specific platelet surface receptors (P-selectin and GPIIb/IIIa) that are important for platelet adhesion to the endothelium and for platelet aggregation. In a clinical study by Rein *et al.* (*27*), subjects consumed one of 3 beverages: water, cocoa containing 897 mg flavanols and related oligomers, or a caffeine-containing beverage. Blood was drawn immediately prior to consumption of the test beverage and post-consumption at 2 and 6 h. Flow cytometry was performed on either unstimulated platelets or after *ex vivo* stimulation using epinephrine or two concentrations of ADP. At 2 and 6 h following the consumption of the cocoa beverage, platelets stimulated with ADP, had significantly less P-Selectin expression. Additionally, there was a trend ($p = 0.053$) for less P-Selectin expression in the unstimulated platelets 6 h after the consumption of cocoa. However, there was no effect on P-selectin in the other treatment groups. Similarly, at 2 and 6 h following the consumption of the cocoa beverage, GPIIb/IIIa expression on unstimulated platelets and those stimulated with epinephrine and ADP (at 20 μM) was significantly decreased. There was also a trend for decreased expression when platelets were stimulated with a higher concentration of ADP (100μM). In contrast, GPIIb/IIIa expression was significantly increased following stimulation with epinephrine in subjects who consumed the caffeine-containing beverage, and there was a trend for increased expression when platelets were stimulated with ADP (20 μM).

In the same study, platelet primary hemostasis was measured using a platelet function analyzer, which measures the ability of platelets to form a hemostatic plug in a collagen membrane. Following stimulation of platelets with collagen and ADP or epinephrine, closure time, or the time that it takes for blood to occlude an aperture, was measured. Compared to the caffeine beverage, when platelets were stimulated with collagen-epinephrine, primary hemostasis, as measured by closure time, significantly increased by 31% at 6 h following the consumption of the cocoa beverage.

Although the mechanisms for the observed anti-platelets effects are unknown, one possible explanation may be the increased production of prostacyclin, an eicosanoid known to inhibit platelet aggregation via increasing cyclic AMP levels. Schramm and co-workers (*28*) have demonstrated that plasma prostacyclin was significantly ($P < 0.05$) higher in subjects following the consumption of 36 g of a high-flavanol (4.0 mg/g) chocolate bar relative to subjects that consumed the same amount of a low-flavanol (0.9 mg/g) chocolate bar (554 ± 37 pmol/L vs. 397 ± 40 pmol/L).

In another clinical investigation by Keevil *et al.* (*29*), 3 juices (purple grape juice, orange juice, or grapefruit juice) were consumed daily in a crossover design of 7-10 days each, and platelet aggregation in whole blood was determined. Ten subjects consumed 5-7.5 mL juice/kg body weight, for an average intake of 450 +/- 120 mL juice/day (about 2 cups). Daily consumption

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of purple grape juice reduced whole blood platelet aggregation in response to collagen by 77% as compared to baseline (p=0.0002). There were no changes following consumption of the other juices. The authors note that the total polyphenol content of the purple grape juice, measured using an indirect and non-specific assay, was approximately 3 times that of the citrus juices (Table I). It is important to note that in addition to differences in the amount of polyphenols contained in these three beverages, the types of flavonoids found in the three beverages are different. It is known that purple grape juice contains flavanols and their related oligomers in addition to other flavonoids, such as flavonols and anthocyanins. In contrast, citrus fruits primarily contain the flavanone class of flavonoids.

In a follow-up study by Freedman *et al.* (*30*), subjects consumed on average ~16 oz. purple grape juice daily for 2 weeks in order to determine if the inhibition of platelet function would be attributed in part to effects on platelet-derived NO release. The consumption of purple grape juice led to a reduction in PMA-dependent platelet aggregation by 19%, and an increase in platelet-derived NO release by 41%. Thus, enhanced release of platelet-derived NO may have contributed to the inhibition of aggregation. While it is unknown how purple grape juice enhances NO release by platelets, NO interacts with superoxide, which may alter the antithrombotic properties of NO. The investigators of this study also measured superoxide, and found that it decreased by 35% after purple grape juice supplementation. This may partly explain the enhancement of NO bioactivity.

Vascular Endothelial Function

The vascular endothelium is a dynamic tissue involved in regulating vasomotor tone, platelet activity, leukocyte adhesion, and vascular smooth muscle proliferation. Vascular tone is regulated by a balance of vasoconstricting agents, such as thromboxane A_2, prostaglandin H_2, and endothelin 1, and vasodilatory agents, to include prostacyclin and nitric oxide, also known as endothelium-derived relaxation factor (EDRF). Nitric oxide (NO) is synthesized from L-Arginine by NO synthase, and while it is the principle compound responsible for vasodilation in arteries, it serves many other vascular functions. Nitric oxide is involved in the inhibition of platelet aggregation, can modulate leukocyte-endothelium interactions by altering cell adhesion molecule expression, reduces monocyte adherence, and finally, inhibits the proliferation of smooth muscle cells.

Endothelial dysfunction is associated with atherosclerosis, and may contribute to the pathogenesis of the disease in the early stages of lesion formation, as well as late in the disease progression. Endothelial dysfunction is detected by activation of endothelium, resulting in release of soluble adhesion molecules such as ICAM-1, VCAM-1, and E- and P-selectin. Impairment in

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Table I. Flavonoids and their concentration in different beverages.

Beverage	*Flavonoid Subclasses*	*Total Polyphenols*		*References*
		GAE	*Chromatography*	
Purple Grape Juice 450 ml	Flavonols, flavanols, anthocyanins, proanthocyanidins	1017		29
Orange Juice 450 ml	Flavanones, flavones	338		29
Grapefruit Juice 450 ml	Flavanones, flavones	387		29
Cocoa 300 ml (18.75 g cocoa powder)	Flavanols, proanthocyanidins		897	26,27
Cocoa 720 ml (36 g cocoa powder)	Flavonols, proanthocyanidins		2610	18
Red Wine 125 ml	Flavanols, flavanols, proanthocyanidins	225	97	5
Black Tea (450 ml (brewed)	Flavonols, flavanols		7335	31

endothelium- dependent vasodilation may indicate the presence of dysfunctional endothelium, and can be measured in the brachial artery using flow- mediated dilation (FMD), a non-invasive ultrasound technique in response to reactive hyperemia. Studies show that endothelial function in the brachial artery is closely related to endothelial function in the coronary artery (*15*).

Flow-mediated dilation of the brachial artery has recently been utilized in a number of studies that assessed the effects of flavonoid- rich beverages, such as black tea and purple grape juice, on vascular endothelial function. In a clinical intervention involving patients with coronary heart disease, Stein *et al.* (*22*) investigated the effects of purple grape juice consumption on endothelial function and susceptibility of LDL to oxidation. This study was unique in that it was the first intervention study to utilize a whole food to investigate the *in vivo* effects of a flavonoid-rich food on endothelial function in human subjects. After daily ingestion of 21 oz. of purple grape juice for 14 days, FMD significantly increased by 4.2 ± 4.4% from an impaired value at baseline (2.2 ± 2.9%) to 6.4 ± 4.7%. The lag time to LDL oxidation also increased significantly by 34.5%. The authors attributed the improvement in endothelial function, as well as the prevention of LDL oxidation, to the short- term ingestion of the purple grape juice. However, one mechanism by which endothelial function was improved could have been due to the decrease in LDL oxidation, since oxidized LDL is associated with endothelial dysfunction (*15*). Improvement in endothelial function could also have been due to the acute effects of consuming the purple grape juice, as the subjects were instructed to consume a dose 2 h prior to the flow measurement, following a 12-h overnight fast. It is unclear whether the totality of beneficial effects by purple grape juice consumption on vascular endothelial function was due to acute or chronic exposure. Follow-up investigations of purple grape juice have reported a decrease in platelet aggregation following consumption (*29*), and an increase in platelet-derived NO release concomitant with a decrease in superoxide production (*30*). Thus, more than one mechanism may be responsible for the ability of purple grape juice consumption to result in beneficial effects on overall cardiovascular health.

In a recent clinical intervention assessing the consumption of black tea for its effects on vascular endothelial function, patients with coronary artery disease consumed black tea and water in a crossover study design for 4 weeks (*31*). The objective was to test the hypothesis that the antioxidant activity of a flavonoid-rich beverage, such as black tea, could reverse endothelial dysfunction. Following both short- and long-term tea consumption, brachial artery FMD was measured 2 h and 4 weeks after consuming tea, respectively, and was found to increase significantly in patients at both time- points. When subjects were tested for vasomotor function 2 h following an acute dose, after consuming tea for four weeks, their response to reactive hyperemia caused a significant increase in brachial artery dilation (10.8 ± 4.4%), greater than after consuming the tea for four weeks alone (9.5 ± 3.6%) (Figure 4). This suggests that the timing of

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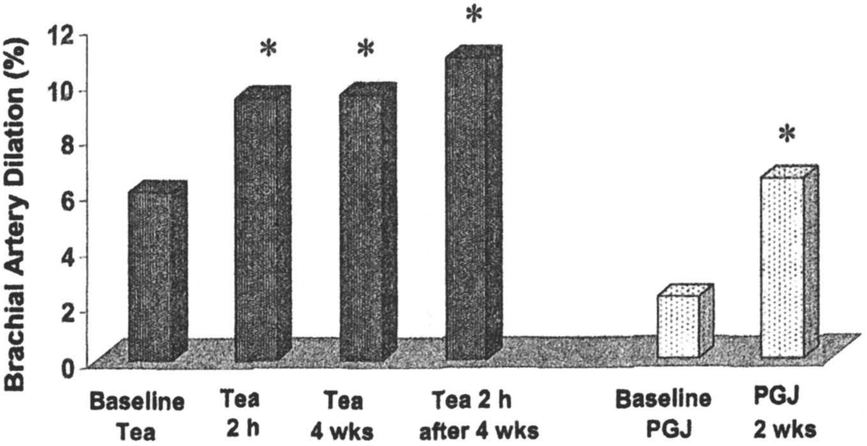

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Figure 4. Improvement of endothelial function with flavonoid-rich beverages. (22, 31.)

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flavonoid intake may prove to be important when assessing effects on the vascular endothelium. The beneficial increase in FMD following daily black tea consumption agrees with the results of daily purple grape juice consumption reported by Stein *et al.* (*22*). Overall, these recent in vivo investigations support the concept that the consumption of flavonoid- rich beverages result in a beneficial increase in vascular endothelial function in the brachial artery.

In order to demonstrate that these *in vivo* effects on vascular endothelium could be due to changes in NO, *in vitro* studies were conducted that resulted in similar findings. For example, changes in EDRF (NO) were seen following the exposure of aortic tissue obtained from New Zealand white rabbits to oligomeric cocoa procyanidins (*32*). In this study, exposure of aortic endothelial cells to cocoa pentamer (an oligomer of epicatechin) resulted in a significant increase in NO synthase activity. Furthermore, the oligomeric cocoa procyanidins (tetramer through decamer) elicited relaxation of aortic tissue that was comparable to or greater than the response to acetylcholine, a control used to relax pre-contracted tissue. Thus, the consumption of a flavonoid-rich food, such as purple grape juice or cocoa, could indeed cause changes in NO production that could translate into beneficial effects on the vascular endothelium.

Bioavailability

It is crucial to demonstrate that flavonoids are bioavailable after they are consumed from flavonoid- rich foods and beverages, which will support the belief that the flavonoids derived from these sources provide part of the biological effects seen *in vivo*. Numerous studies have demonstrated the bioavailability of flavonoids from plant-derived foods and factors affecting their bioavailability, which have recently been reviewed (*33,34*). As discussed previously, Wang and co-workers (14) demonstrated that there is a significant dose-responsive increase in the flavanol, epicatechin, in plasma 2 h after the consumption of increasing amounts of flavanol-rich chocolate (Figure 2). The findings were similar to those of Richelle and co-workers (*19*), who monitored the plasma kinetics of epicatechin over an 8 h time period after volunteers consumed two different doses of black chocolate. These investigators reported that there was a dose-response effect in plasma epicatechin after consumption of black chocolate, with plasma epicatechin reaching its peak 2 h post consumption. Similar studies with red wine and black tea also showed that the flavanol, catechin, is bioavailable following consumption of these beverages. For example, Donovan *et al.* (*35*) found that 1 h after consumption of 120 mL of red wine (providing 35 mg of catechin), plasma levels of catechin and its conjugates reached a maximum level, decreasing to baseline levels by 8 h post consumption. Catechins in black tea have also been assessed after repeated consumption over the period of one day (*36*). These investigators found that following the consumption of four servings of tea (providing 400 mg of tea catechins per serving), plasma concentrations of tea catechins increased during an 8 h period in which the tea was consumed. Taken together, these studies demonstrate that the flavanols from foods and beverages such as chocolate, red wine, and green tea are bioavailable, and may indeed contribute to the biological effects previously discussed. There is a need however, to further assess the bioavailability of procyanidin oligomers in foods and beverages.

While the bioavailability of the flavanols has been well documented, there is still limited information concerning procyanidin absorption. Radiolabelled techniques have indicated that the procyanidins are bioavailable, though these studies did not demonstrate whether the procyanidins were intact or depolymerised prior to absorption. Recently, Holt *et al.* (*37*) reported that cocoa procyanidin dimer B2 (epicatechin-(4β-8)-epicatechin) can be detected in the plasma of human subjects within 30 min of consuming a cocoa beverage, reaching a maximum concentration in the plasma 2 h after consumption.

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Conclusion

In summary, clinical data from intervention studies suggest that significant cardiovascular health benefits may be derived from regular consumption of beverages containing flavanols and their related procyanidin oligomers. These potential benefits include enhancing plasma antioxidant capacity, decreasing susceptibility of LDL to oxidation, decreasing platelet aggregation, and improving vascular endothelial function. In addition, it is important to emphasize that the effects seen *in vivo* have been demonstrated *in vitro* as well. However, these vascular effects appear to be transient in nature, with a return to pre- consumption values 2-8 h after consumption. While it is recognized that the beneficial health effects observed in studies to date could indeed be due to the flavonoid content of the interventions, (i.e., red wine, green and black tea, purple grape juice, and cocoa), additional research is needed to determine the real potential of flavonoid- rich foods and beverages to benefit long- term cardiovascular health.

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Chapter 19

Proanthocyanidins, Bisflavanols, and Hydrolyzable Tannins in Green and Black Teas

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The content of proanthocyanidins and bisflavonols in green and black tea samples was determined. The average content of proanthocyanidins (sum of 16 different compounds) in green tea was 0.84 % while that in black tea was considerably lower (0.5 %). However, the reverse was true for the bisflavanol (2 compounds) content, which was 0.05 % in green teas and 0.65 % in black teas. The hydrolyzable tannin strictinin (1-O-galloyl-4,6-(-)-hexahydroxydiphenoyl-β-D-glucose) was determined using an HPLC-ESI-MS/MS procedure and alternatively by HPLC-UV after polyamide clean-up. Strictinin contents in tea samples varied between 0.2 and 1.5 g/kg. Analysis of a fermentation series (green tea, 30, 60, 90 and 120 min fermentation of the same batch of tea leaves) showed a decrease with increasing fermentation time. The *in-vitro* antioxidant activity of selected tea proanthocyanidins (ABTS-method) was between 4.38 (EGC-EGC: epigallocatechin-4ß 8- epigallocatechin) and 7.66 (EGCG-ECG: epigallocetechin gallate-4ß 8-epicatechin gallate) mmol/mmol Trolox.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

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Flavonoids from green and black tea and other sources have attracted much interest. Catechins, especially epigallocatechin gallate (EGCG), theaflavins and flavonol glycosides are thought to be responsible for antioxidative properties in tea (e.g. *1-5*). Flavonol glycosides (FOG) have activities against myocardial infarction, stroke and several types of cancers. The properties of the compounds, including their absorption characteristics, have recently been reviewed (*1-5*). Data for the contents of catechins and other groups of flavonoids in tea are readily available (e.g. *1*, *6-8*). However, there is little information available on the contents of proanthocyanidins, bisflavanols and hydrolyzable tannins in tea.

Determination of Proanthocyanidins

Proanthocyanidins and bisflavanols are oligomeric flavanols which might consist of the same monomeric units. The difference is the type of the interflavonoid bond, which is usually 4-8 or 4-6 for proanthocyanidins and 2'-2' in case of bisflavanols as shown in figure 1.

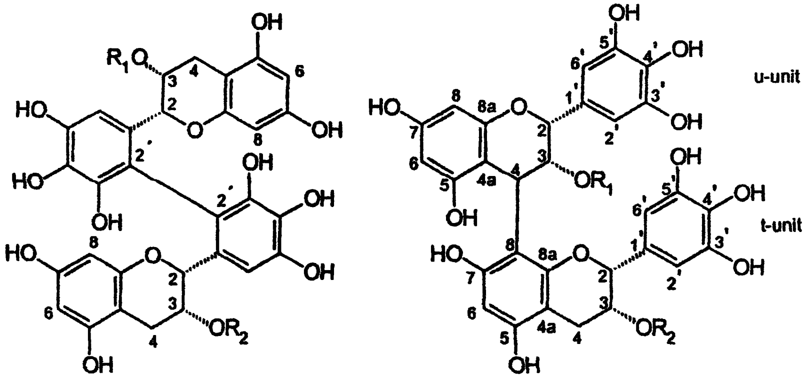

Figure 1. Bisflavanols and proanthocyanidins

A number of proanthocyanidins and bisflavanols have been isolated from fresh tea leaves, green tea and oolong tea samples (*9-12*), but data on their contents are scarce. In one study the amounts of four proanthocyanidins and bisflavanols A and B have been detected in fresh tea leaves and their changes during fermentation studied (*13*). The amounts of individual compounds were between 0.8 and 2.1 g/kg fresh weight. Galloylated compounds fast degraded during fermentation while the amounts of non-esterified compounds were about the same after 12 h of fermentation. The amounts of bisflavanols A and B were 1.4 and 0.6 g/kg, respectively (*13*).

Methodology

The method used for separation and structural elucidation of compounds has been described elsewhere (*8, 14, 15*). Briefly, proanthocyanidins were extracted from ground leaves (2.5 g) twice with 100 mL 75 % acetone (aq.) in case of exhaustive extractions. After removing the organic solvent the residue was made up to 50 mL with water. The extract (5 mL) was brought to a conditioned polyamide cartridge (1 g polyamide). After washing (methanol, acidified methanol, 20 mL resp.) the compounds were eluted using 75 % actecone (aq.). This extract was dried, made up to 10 mL with 5 % acetonitrile and separated by RP-HPLC. For calibration purposes self-isolated and characterized proanthocyanidins were employed (*15*).

The specifics of methods used were as follows: recovery of GC-4α-8-EGCG (gallocatechin-4α-8-epigallocatechin gallate) was 86 % and that for EGCG-4β-8-ECG was 81 %. The RSD was between 2.1 and 10.7 % depending on the compound.

Table I. Proanthocyanidins and bisflavanols in green and black tea samples

	Green tea	*Average*	*Black tea*	*Average*
Proanthocyanidins	0.13 – 1.89	0.84	0.10 – 0.98	0.50
Bisflavanols	0.01 – 0.11	0.05	0.33 – 0.81	0.65
Sum	-	0.90	-	1.15
proportion of bisflavanols	0.9 – 12.7*	5.9*	40.2 – 77.1*	59.7*

Note: Units are g/100 g; * = % from the sum of both groups of compounds

Table I gives an overview of the contents of proanthocyanidins and bisflavonols in green and black teas (no. of samples was 29 green and 9 black teas).The average amounts of proanthocyanidins and bisflavanols in green and black teas was around 1 % dmb which is surprisingly high. The ratio of proanthocyanidins and bisflavanols might serve as an analytical citerion for the discrimination between green and black tea samples. In black tea samples the proportion of bisflavanols determined to the sum of both groups of compounds was around 60 percent. In green tea this proportion was usually below 10 % which supports the statement that the content of proanthocyanidins does decrease during fermentation while the content of bisflavanols is increased.

To give more detailed information on the contents of individual compounds average contents are shown in Figure 2. As can be seen in black tea the major compound was bisflavanol A while in green teas EGC-4ß-8-EGCG and GC-4ß-8-EGCG were most abundant.

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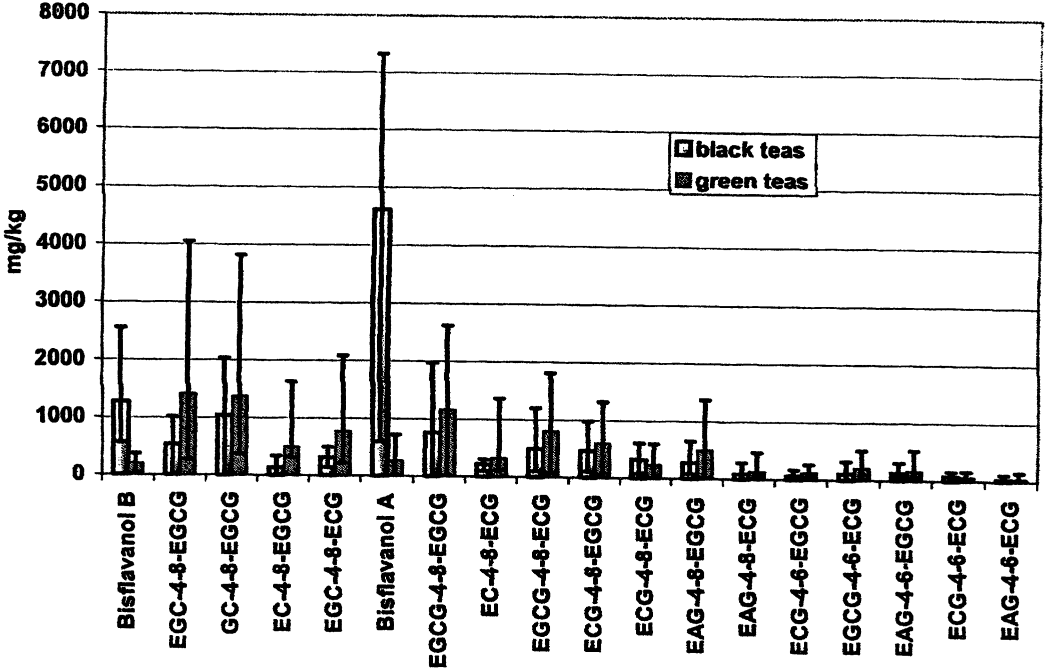

Figure 2. Average contents of bisflavanols and proanthocyanidins in green and black tea samples

Extraction of proanthocyanidins in tea brews

To assess the extraction efficiency green tea beverages were prepared in the following matter: 1.5 g of tea were brewed with 100 mL of water (80 °C) for 3 min, then filtered. Figure 3 gives the extraction efficiencies of proanthocyanidins in % compared with "total extract" (extraction with aq. acetone, see above). As can be deduced from the figure the extraction of the 4-6 compounds was in general lower compared with the 4-8 compounds. Figure 3 shows the extraction yield of Chinese green tea samples.

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Determination of strictinin (1-O-galloyl-4,6-(-)-hexahydroxydiphenoyl-β-D-glucose)

Strictinin was identified as a constituent of green and black teas by Japanese groups (9,13). We also found strictinin along with 1-O-digalloyl-4,6-(-)-hexahydroxydiphenoyl-β-D-glucose in green and black tea samples after the clean-up procedure described above. It was not possible to determine strictinin along with the proanthocyanidins as it was present in 2 fractions of the polyamide clean-up. Consequently, a different method of determination had to be developed. The first approach was a HPLC-ESI-MS/MS procedure which had the advantage that no sample clean-up was necessary. Briefly, the instrument was run in the negative mode and the mass 633 $[M-H]^-$ was isolated and fragmented to yield a fragment at m/z 301 ($[\text{ellagic acid-H}]^-$). The corresponding mass track was used for quantification purposes. Alternatively, the clean-up procedure by polyamide was modified (both fractions were pooled and analyzed). The latter has the drawback that a lengthy gradient system has to be employed, which is time-consuming.

A study with a fermentation series showed that the amount of strictinin decreased during fermentation; strictinin takes part in the thearubigin formation (i) or that the compound is irreversibly bound to proteins during the firing process (ii). Figure 5 shows the decrease of the strictinin content with increasing fermentation time. The determination of the digallate was not possible due to the fact that the standard isolated was not pure enough, moreover, there are probably chemical reactions in solution leading to the formation of at least 2 peaks in the chromatogram. A method to isolate a pure standard of strictinin-3'-gallate via HSCCC is in progress. This pure standard will also be useful in establishing the behavior of the compound in solution.

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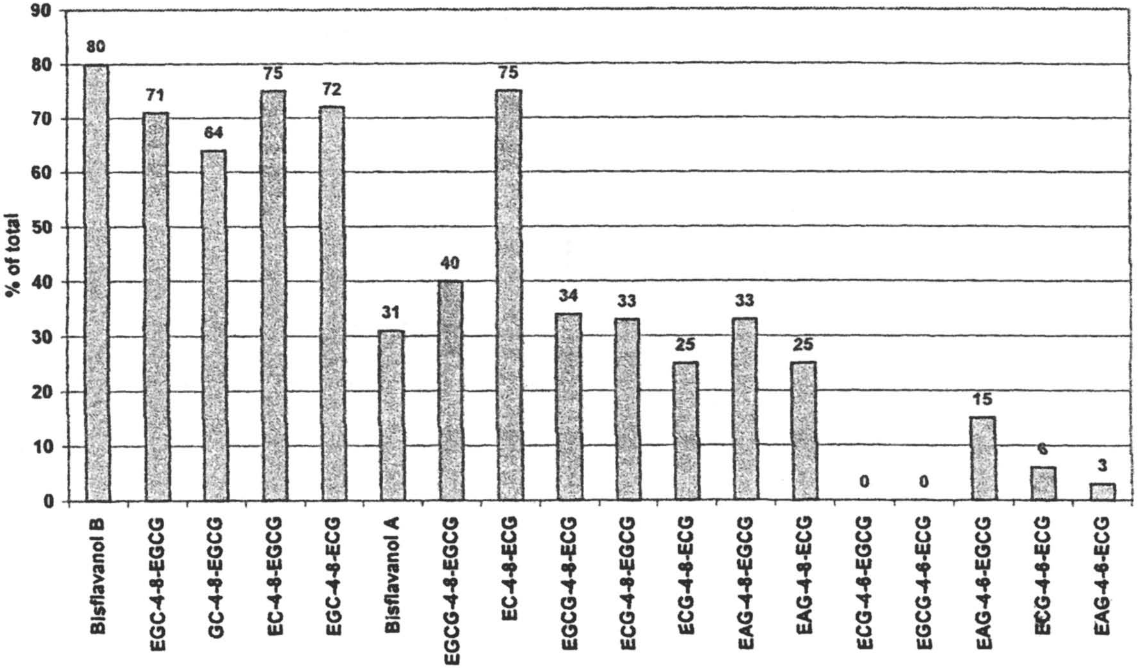

Figure 3. Extraction of proanthocyanidins and bisflavanols in tea brews as compared with an exhaustive extraction, details see text

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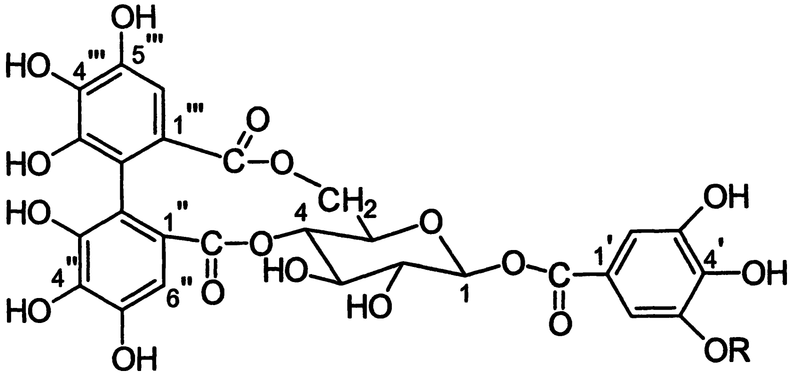

Figure 4. Strictinin (R=H) and its 3'- gallate (R = galloyl)

Table II. Strictinin in selected green and black teas

Sample origin	*type*	*strictinin** *(LC-MS/MS)*	*strictinin** *(LC-UV)*
Darjeeling	black	1285	1525
Assam	black	876	690
Indonesia	black	694	574
China	green	*1027*	1271
SC 12/28**	green	277	n.d.
CHM 61/60**	green	220	n.d.
SF 186**	green	219	n.d.
S 15/10**	green	209	n.d.
CG 28 U 864**	green	203	n.d.
CG 28 V 929**	green	177	n.d.

* Data given in mg · kg^{-1}; ** Kenyan samples

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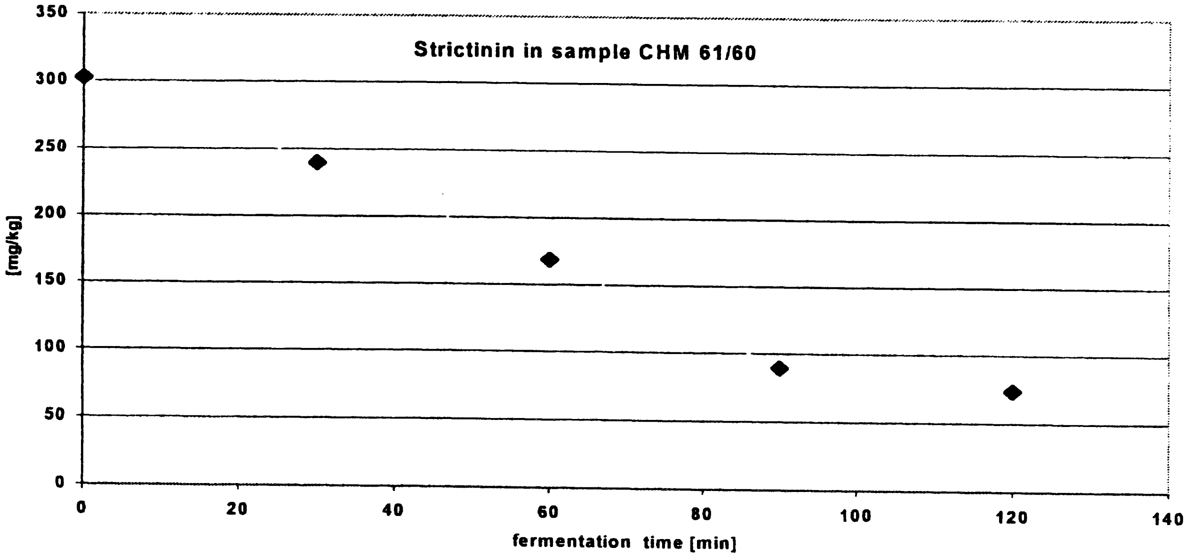

Figure 5. Strictinin in a fermentation series from a Kenyan tea sample

Strictinin has also been analyzed in a series of commercial tea samples. We analyzed samples from aqueous tea brews and also from acetone extracts. Selected results are shown in Table II for acetone extracts. The results of LC-MS (without clean-up) and LC-DAD (including polyamide clean-up) are not quite the same but at least comparable.

Currently the reason for the difference between both methods is under investigation. This is a prerequisite for a proper validation of the methods which is also in progress.

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Trolox equivalent antioxidant capacity (TEAC) data of proanthocyanidins and strictinin from tea

Tea is one of the beverages with a high antioxidant capacity *in-vitro*. To get an idea about the contribution of the compounds in question the antioxidant activity was measured using the TEAC test (*16*). The data suggest that the dimers have a relatively high antioxidative potential, but it was less than the addition of the monomer units. Table III gives the data in comparison with some

Table III. Antioxidant activity of tea constituents in comparison with literature data (*17,18*)

	TEAC [mmol Trolox/mmol]	*TEAC (literature data) [mmol Trolox/mmol]*
vitamin C	n.b.	1.0 +/- 0.02[17]
vitamin E	n.b.	1.0 +/- 0.03[17]
epicatechin	2.97	2.5 +/- 0.02[17]
catechin	2.82	2.70[18]
epigallocatechin	3.46	3.8 +/- 0.06[17]
epigallocatechingallat	4.11	4.8 +/- 0.06[17]
epicatechingallat	3.84	4.9 +/- 0.02[17]
procyanidin b-1	-	4.26[18]
procyanidin b-3	-	4.79[18]
EGC-4β→8-ECG	4.38	-
EGC-4β→8-EGCG	4.90	-
GC-4α→8-EGCG	5.60	-
EGCG-4β→8-ECG	7.66	-
EGCG-4β→8-EGCG	6.68	-
bisflavanol A	6.33	-
strictinin	5.50	-
quercetin	3.88	3.84[18]
rutin	2.82	2.4 +/- 0.06[17]

data from the literature. Taking those data into account it is likely that the contribution of the dimeric and trimeric proanthocyanidins and bisflavanols to the antioxidant capacity of tea beverages is the same order of magnitude as the flavonol glycosides.

Strictinin has also a pretty high antioxidant capacity but the amounts in tea sample are usually low compared with the proanthocyanidins/bisflavanols.

Acknowledgements

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Chapter 20

Soy-Based Nutritional Beverages

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The FDA's approval of the health claim for soy protein has triggered tremendous interest in developing soy-based products. Of these, ready-to-drink nutritional beverages are of most interest. In the course of developing nutritional beverages, several criteria need to be considered. These are target market segments, consumer age group, special nutritional requirements, shelf life requirement for the distribution, nutritional profile of macronutients, vitamin and mineral supplements and finally, packaging and processing methods. In this overview, discussion will be focused on the design of nutritional beverages targeting different consumer groups. These beverages are designed for general well being, balanced nutrition, generation Y (the thirties), generation X (the forties), the baby boomers, weight loss and meal replacement, geriatric and medical nutrition. Given the merging trend of nutraceutical with the mainstream foods, soy is the prime choice of ingredient. Along with other nutraceuticals, soy protein based products will soon move to center stage for consumer foods. Compatibility and interaction of these ingredients and the effects of processing are also discussed.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

The FDA's approval of the soy health claim (*1*) has stirred considerable research and product development activities around the world. With advanced food technology and processing techniques, various protein products allow manufacturers to develop foods that offer excellent taste, texture, mouthfeel, flavor and eating quality with distinct nutritional and health benefits.

Soybeans have 42% protein, 20% fat, 20% carbohydrate, 5% hull and 13% ash and water. After oil extraction and desolventization, defatted soy flour has 50% protein. By further refining to remove more carbohydrates 70% protein soy flour is obtained (soy concentrate). After more elaborate processing, a product with ≥90% protein on a dry weight basis known as soy protein isolate is produced (isolated soy protein).

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Protein Quality

Not all proteins are created with equal nutritional values. For Supro® isolated soy proteins (DuPont Protein Technologies, St. Louis, MO), a tremendous amount of research effort has been devoted to understanding the effect of processing on the product quality and also on its effects on nutritional value and protein utilization. Each soy protein product is specifically designed and manufactured to meet a particular application requirement.

During the last three decades, we have studied the protein quality and food applications for human consumption, heart disease, cancer, women health, sport nutrition, diabetes and weight management. Traditionally, protein efficiency ratio (PER) has been used as the standard measure of protein quality. A protein with PER of 2.5, using casein as the reference protein, is considered a complete protein (*2*). However, with the advanced research and understanding of biochemical science and nutrition, we learned that sulfur-containing essential amino acids are interconvertible and the human body has unique mechanism in conserving and recycling these sulfur-containing amino acids. Further, PER was established based on rat studies. In comparison to rat, human have much less hair and thus much lower requirements for sulfur-containing amino acids (*3*).

Therefore, the current accepted method for evaluating protein quality by the United States Food and Drug Administration (FDA) is the Protein Digestibility Corrected Amino Acid Score (PDCAAS). The PDCAAS takes into account the food protein's essential amino acid content, its digestibility, and its relevance to human needs. The highest possible score of PDCAAS is 1.00. In Table I, the PDCAAS scores from various food protein sources are presented (*2*). As demonstrated in the table, ISP has a PDCAAS score of 1.00, comparable to the quality of meat, milk and egg proteins.

The amino acid requirement pattern of the needs of a 2 to 5 year old child, 10-12 year old child and an adult is shown in Figure 1, which represents the most demanding amino acid requirements of any group, except infants. As shown in the darkened histograms, Supro® brand isolated soy proteins meet the requirements of all the three age groups.

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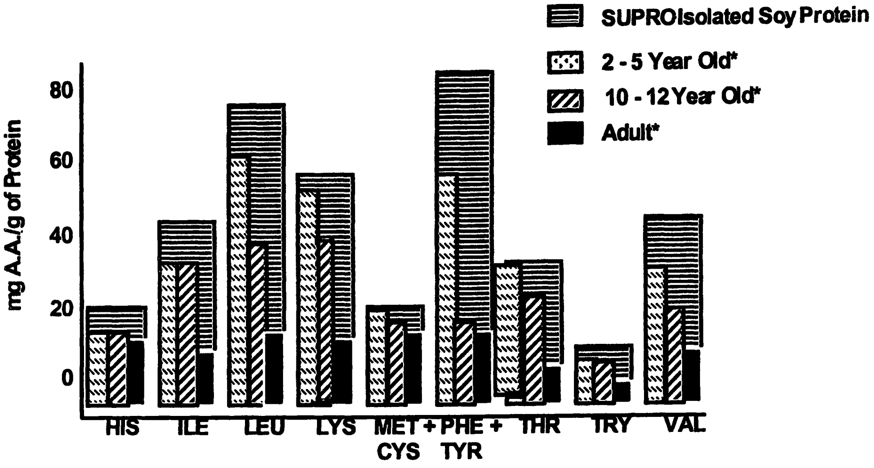
Table I. Protein Digestibility Corrected Amino Acid Scores

Product	PDCAAS
SUPRO® Isolated Soy Protein	1.00
Casein	1.00
Egg White	1.00
Skim Milk Powder	1.00
Whey Protein Concentrate	1.00
Beef Protein	0.92
Pea Flour	0.69
Kidney Beans (Canned)	0.68
Pinto Beans (Canned)	0.63
Whole Wheat	0.40
Wheat Gluten	0.25



* Suggested Pattern of Requirements (FAO/WHO/UNU, 1985).

Figure 1. FAO/WHO/UNU Essential Amino Acid Requirement Patterns
(Reproduced from reference 4. Copyright 1985.)

Calcium Bio-availability

One of the major concerns of using soy protein in a milk type nutritional beverage is that the calcium content of soy is relatively low and exogenous sources of calcium are required to bring up the calcium level. When supplementing calcium, the two major challenges are the bioavailability and the homogeneous suspension of calcium in the finished beverage. The results from an animal feeding study indicate that the calcium is biologically available from calcium fortified soy beverages and calcium neutralized ISP is equal to the calcium avalable from calcium carbonate and nonfat dry milk (Table II).

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Table II. Calcium Bio-availability using rat as experimental model

	Relative Bioavailability, %	
	Serum Calcium	Bone Calcium
Calcium Carbonate	100	100
Di-Calcium Phosphate	95 – 109	100 – 112
Tri-Calcium Phosphate	91 – 107	94 – 106
Calcium Oxide	90 – 105	97 – 110
Skim Milk Powder		108 – 124
UHT Ca-Fortified Soy Beverage		97 – 113
Isolated Soy Protein + Whey		99 – 114
Ca-Neutralized ISP		103 – 119

Source: Adapted from reference 5.

Both calcium fortified soy beverages and neutralized ISP, showed excellent calcium bioavailability. To ensure that all the fortified calcium in beverages are available to the consumer, it is necessary to suspend homogeneously all the beverage calcium. Through DuPont Protein Technoliges's (referred as "DPT") patented technology (US Patent 4,642,238), calcium in the soy beverages is much better suspended. With this patented technology, more than 70% of the added calcium was suspended throughout the shelf life of beverages, only 20-40% suspension of calcium was achieved using the conventional gum technologies (*8*).

The ISP's with stable calcium technology are called Supro Plus® ISP (DuPont Protein Technologies, St. Louis, MO). The bioavailability of calcium from Supro Plus® protein was equal to that supplemented calcium salts (Table III) (*6*). Thus, the bioavailability of calcium in Supro Plus® ISP is as good as commercial sources of calcium and cow's milk (*5*).

Table III. Calcium Bio-availability of Isolated Soy Based Diet

Calcium Source	Unit	None	SUPRO Plus	Ca Salt
Calcium Intake	mg/wk	28.5	646	783
Bone Mineral	g	0.04	0.31	0.34
Bone Density	g/cm^2	0.07	0.22	0.23
Serum Ca	mg/ml	7.9	12.60	11.20
Serum PTH	pmoles/L	235	135	129

Source: Adapted from reference 6.

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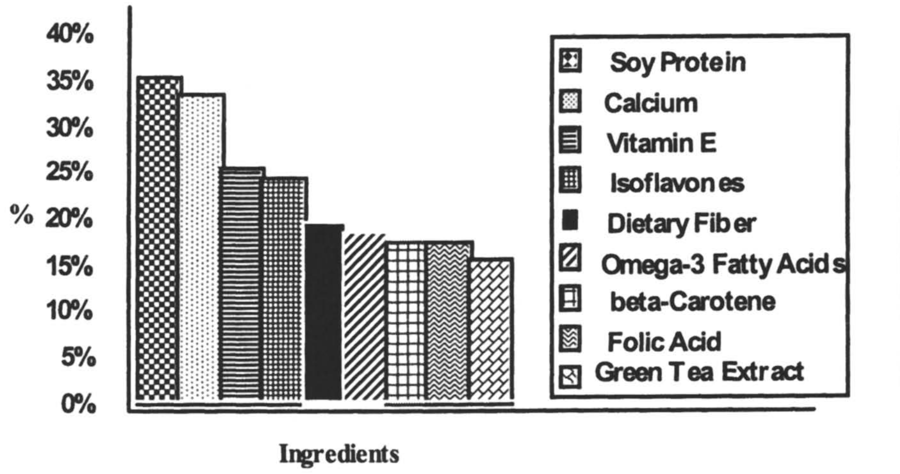
Soy Ingredients for Product Development

Twenty-five grams of soy protein a day reduces the risk of coronary heart disease. There are a series of bioactive compounds in soy, namely globulin, peptides, amino acids, phytic acid, saponins, isoflavones and protease inhibitors. No wonder that *Prepared Foods* (*8*) elected soy proteins, along with the other eight ingredients (Figure 2), as the top choice of ingredients by food product developers.



Source: Prepared Food, November, 2000

Figure 2. Top Ranking Ingredients
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Beverage Applications

When using ISP in the development of nutritional beverages, the most important part of the application is dispersion and hydration of proteins. Only when the protein is properly dispersed and hydrated, can the expected functionality be expressed to create a stable and palatable beverage.

Dispersion and Hydration

A well-dispersed and hydrated protein solution with a homogeneous phase is the first requirement for formulating good tasting and stable ready-to-drink (RTD) beverages. Isolated soy protein powder needs to be dispersed into individual particles in the processing water without any lumps. Lumps may be present if product is not properly dispersed. Time, temperature, shear energy, pH and ionic environment are the five factors affecting hydration of soy proteins. At constant temperature, prolonged hydration time results in a good protein hydration. When temperature is increased to180°F, proper hydration is reached at a relatively shorter mixing time, say, 15 min.

Higher shear energy facilitates hydration. However, care should be taken not to incorporate too much air into the protein slurry. Entrapped air in the beverage solution impedes the homogenization efficiency.

At around pH 4.5, the isoelectric point of soy protein, ISP is least soluble (Figure 3). At pH's distant from the isoelectric point, improved protein hydration can be obtained. For organoleptic and functional reasons, pH range from 6.8 to 7.2 is most desirable for a low acid nutritional beverage.

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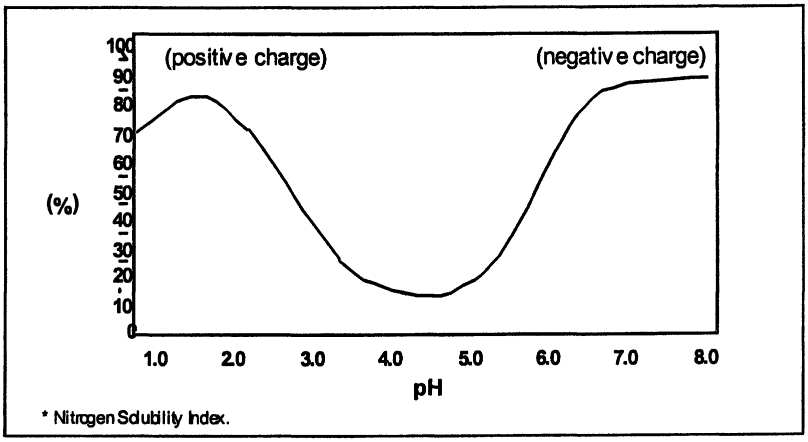

* Nitrogen Solubility Index.

Figure 3. Solubility of Isolated Soy Protein as A Function of pH*

High ionic strength also reduces the solubility of protein.. The solubility of the protein drops to 60% when the ionic strength of sodium chloride reaches 0.1M. Further increases in ionic strength increases the solubility. Somewhat presumably due to the charge effect.

Divalent ions such as calcium, magnesium and zinc link to the negatively charged carboxyl ends of the protein and thus cause proteins to aggregate (Figure 4). However, a nutritional beverage requires the fortification of all three divalent ions. Chelating agents such as citrates and phosphates are required to make these ions unavailable to interact with proteins.

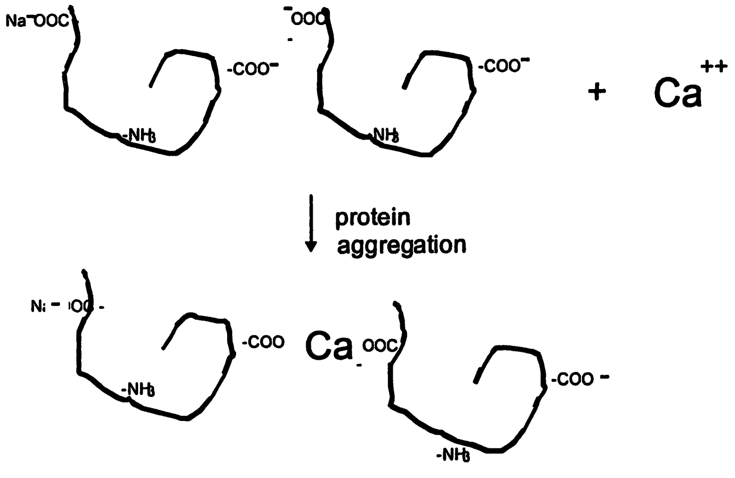

Figure 4. Divalent Ions Promote Protein Aggregation

An experiment was conducted (*9*) to determine the required amounts of either citrate or sodium hexametaphosphate to chelate a known concentration of calcium or magnesium, it was found that sodium hexametaphosphate was a more powerful chelating agent than citrate. For citrate, almost a one-to-one ratio of concentration of sodium citrate to calcium ion is required to chelate the free calcium. For sodium hexametaphosphate against magnesium ion, this ratio was only about 0.16.

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Gum stabilizers are also commonly used to help suspend the protein and minerals in beverage. These gums are carrageenan, colloidal microcrystalline cellulose gel, cellulose gum, pectins and sodium alginates. Gums help build intermolecular bonding through electrostatic linkage, creating interstitial matrix making the protein movement in the fluid less freely or just by increasing the viscosity of the beverage to slow down gravity movement.

In summary, to achieve a good hydration and thus a good functionality of protein in the finished beverage, we recommend that a soft water (total hardness less than 40 ppm expressed as calcium carbonate) or even de-ionized water be used. First dissolve the entire buffer salts completely before adding proteins. When adding proteins, raise the water temperature to 120°F with the mixing speed set at high. For production practice, a powder-dispersing device such as Tri-blender or Likwifier is highly recommended to use. These type of devices provide vigorous mixing and re-circulation of the dispersing fluid back to the high-shear-dispersing pump, which results in a lump free protein dispersion. The stabilizers, due to their minute quantities, can either be added before or after protein hydration. Since the quantities of the stabilizers are usually very small, an equal amount of sugar can be used to make a pre-blend to help with dispersing. Once protein and stabilizers are dispersed properly, the solution should be allowed to continue mixing at medium speed for 15 min. Medium speed used is to minimize any unnecessary incorporation of air, which, if not removed, may decrease the homogenization efficiency.

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Protein Selection

When developing a soy based nutritional beverage, several considerations such as flavor and mouthfeel, suspension and emulsion stability, nutritional and health benefits, viscosity, color, mineral and vitamin fortification and micro biological purity all need to be considered depending on the development criteria required. A few examples demonstrating these criteria are presented below.

When carbohydrates are used together with proteins, the choices of proteins and types of carbohydrates determine the final color of the finished beverages. In Figure 5, the upper row is the combination of caseinate with maltodextrin and corn syrup solids with various degrees of dextrose equivalent (DE). The lower row is the counterpart of caseinate using Supro Plus® 651 (an ISP protein product with suspendable calcium in the protein matrix supplied by DPT).

It is clearly demonstrated that soy protein has fewer interactions in the browning reaction and thus much whiter finished beverages result.

The viscosity has influence on the stability of the emulsion, the mouthfeel of the finished beverages, and the flowability of the beverage through the tube feeding. Depending on the protein content of the finished beverages, proteins with different viscosity can be chosen for the purpose. At lower protein content, i.e., $\leq$ 6.25g protein/8oz serving, proteins with slightly higher viscosity should be used to provide better mouthfeel and creaminess. While for balanced nutritional beverages, i.e., $\geq$12.5g/8oz serving, a lower viscosity protein should be used.

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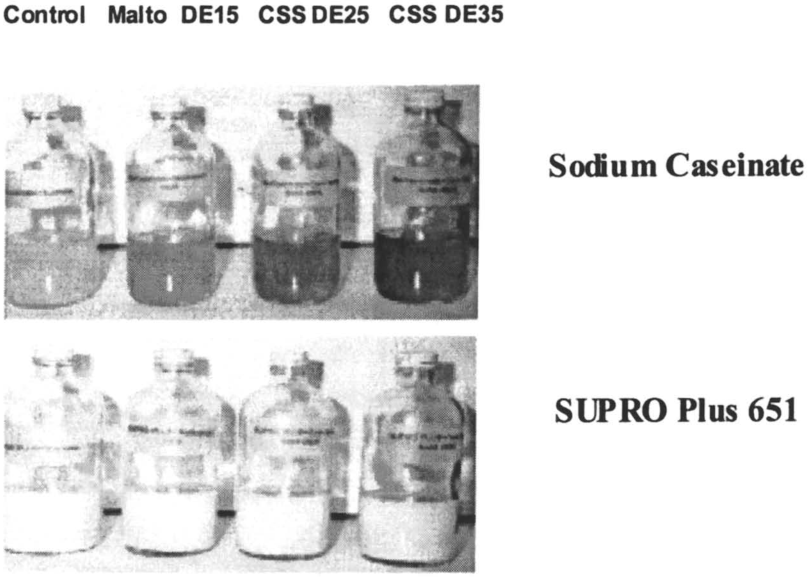
Control
Malto DE15
CSS DE25
CSS DE35
Sodium Caseinate
SUPRO Plus 651

Retorted at 121°C for 15 Minutes Dr. XiolinHuang, DPT internal data

Figure 5. Maillard Reaction of Sodium Caseinate or Supro Plus® 651 with Different Carbohydrates
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Emulsifying properties of soy protein are very important for a stable beverage emulsion. For a high energy beverage and nursing home feeding program, as high as 2.0 Kcal/mL of beverage are quite common to provide between 360 to 480 Kcal per 8oz serving of beverage. Other than carbohydrates and proteins, the major cost-effective energy source is fats and oils. In Figure 6, the oil emulsion capacity of six different Supro® proteins are presented. The oil holding capacity is defined as the milliliters of oil can be held by one gram of protein in a defined concentration.

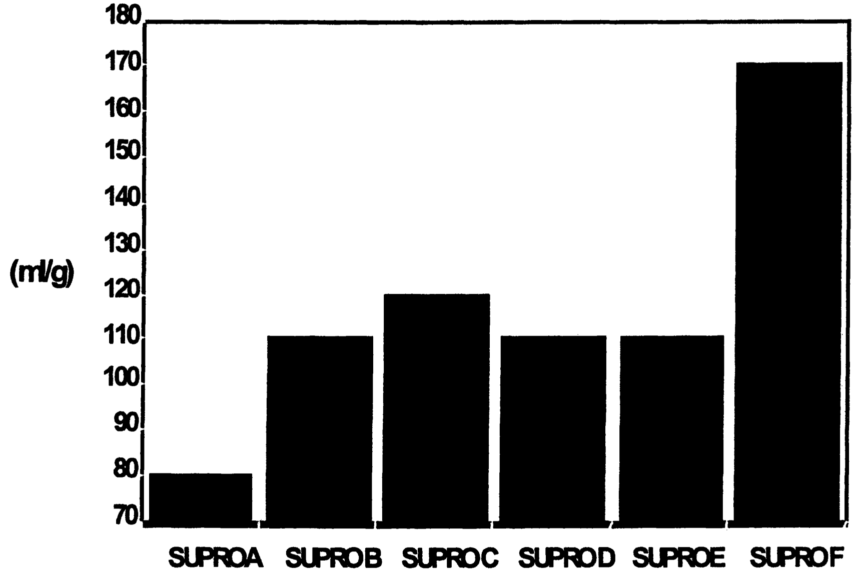

Figure 6 Emulsion Capacity of Various Types of Supro® Proteins

To meet different product development needs, DPT has Supro® proteins with PDCAAS scores of 1, Surpo Plus® proteins with stable calcium, Supro XT® proteins with improved flavor, dispersibility, managed minerals and controlled viscosity and SuproSoy with a certified level of total isoflavone at 3.4mg/g protein. Sharing the product development needs with the ingredient supplies is the key to a quick turn around for timly commercial success. Trust needs to be built up between the manufacturer and the supplier to achieve such a relationship.

Considerations for Beverage Development

In the process of developing successful commercial nutritional products, it is important to consider the nutrition profiles, the economy, the functionality and the health benefits. For nutritional consideration, we can divide it into macronutrients and micronutrients. For macronutrients, we consider calorie, fat,

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carbohydrate & sweeteners. For micronutrients, we refer them to vitamin/mineral premix. The forms of the minerals do have significant impact on the color, flavor and taste of the finished beverages. For vitamins such as vitamin C, a significant level of overage should be built into the formulation to deliver the claimed values. This is a very specialized area and we recommend working with vitamin/mineral suppliers and communicate to them your processing conditions to ensure a reliable delivery of micronutrients

For health oriented beverages, often, certain nutraceuticals are required to address certain market segments. This in general adds complication to the beverage system. For example, eicosapentaenoic acid (EPA) & docosahexaenic acid (DHA) in fish oil and conjugated linoleic acid (CLA) are very unsaturated and are thus very prone to oxidation. The oxidative decomposition products from these oils are notorious in odors and once developed are almost impossible to remove them completely. Green tea extracts and other polyphenolics of plant origins have an inherent property of precipitating proteins and add to the beverage instability. In the presence of ferrous or ferric ions, tea polyphenols react with iron to cause discoloration.

For economic reason, one needs to consider the quality, application target, and consistent supply. Only all the requirements are fulfilled can the economy be considered. The health benefits of soy, the versatility of different soy ingredients for product development and in view of the recent rise in dairy prices, it justifies the consideration of this versatile ingredient.

For commercial development, next to considered are the target market segment, ingredients decoration and labeling requirement (whether natural is required), package requirement, and the processing facilities. Finally and most importantly, the target date for commercial launch. Every activity caters to this date set.

Criteria for A Good Tasting Products

As reported by Tiax, LLC (*11*), successful food products in the market generally possess the following criteria: early impact of appropriate flavors, pleasant mouth sensation, full body of highly blended flavors, rapid disappearance of flavors on swallowing and absent of unpleasant aftertastes. In an attempt to achieve all these criteria, we examined the flavor attributes associated with a soyobased products as given below.

Characteristic Flavors Associated with Soy

Beany, cereal-like, astringency, harsh, mouth drying and throat catching, slightly bitter if the soy protein is partially hydrolyzed, strong bitter and savory

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notes when highly hydrolyzed, and grassy and green mainly due to lipid oxidation are the most commonly described soy flavor attributes. To remove or suppress all these undesirable flavors, flavor houses are working with different varieties of masking agents. An ideal masking agent is the one that can mask the undesirable flavor without producing any flavor characters of its own. A universal one-for-all masking agent is still the challenge for flavor houses as well as product developers.

Flavors Compatible with Soy

Numerous good flavored soy beverages have been introduced to the market with favorable response. It is our experience that certain flavors work better with soy than others. These flavors either deliver a brown note or go relatively well with soy. For brown flavors, we recommend hazelnut, English toffee, coffee latte, chocolate, caramel, rum raisin, and malt. Other flavors such as cream, almond, passion fruit, peach, apple, cereal, coconut, banana and honeydew melon and cantaloupe also go very well with soy–based beverages.

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Summary

With some care and following of the basic rules recommended above, good tasting soy-based nutritional beverages can be successfully developed. The flavor development work involves the covering or masking of the undesirable soy notes, conducting a high and low UHT production with the target flavor system, allowing for neuttal beverages 10 to 14 days holding period after UHT processing, tasting and adjusting the flavor to a desirable level, and confirming the selected dosage with repeated UHT productions. Over flavoring should be avoided due to the disproportional concentration of certain chemical components rendering the product with a strong chemical taste which would be unacceptable. As a rule of thumb, a mix flavor is always better than a single flavor.

Acknowledgement

The authors would like to take this opportunity to thank Ms. Patsy King and Ms. Katherine Reinecke for their skilled editing efforts on the text and Dr. Xiaolin Huang for use of some of his data and pictures.

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Chapter 21

Antioxidant Characterization of Caseinophosphopeptides from Bovine Milk

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Bioactive peptides have been isolated from casein hydrolysates and shown to have the affinity towards binding divalent metals such as calcium, zinc, copper, manganese and iron. The purpose of this study was to determine if tryptic digests of bovine milk casein deriving caseinophosphopeptides (CPP) carries the potential to hinder metal induced oxidation reactions through strong chelation with divalent metal catalysts. Using site-specific and non site-specific deoxyribose assays, CPP were found to significantly lower the level of secondary oxidation products and hence the extent of iron-mediated oxidation. In addition to secondary antioxidant activity, CPP also interacted directly with and stabilized free radicals such as ABTS radical, displaying yet another extraordinary property as a potential antioxidant.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

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Caseinophosphopeptides (CPP) are bioactive peptides derived from tryptic hydrolysis of bovine milk protein, casein. They are named phosphopeptides due to the presence of a high concentration of phosphorylated serine residues therein. The extent of phosphorylation is highly dependent on the type of caseins (α_{s1}, α_{s2}, β or κ) from which the peptides originate, ranging from having only 1 phosphate group in κ-casein to over 13 in α_{s2}-casein (*1*). A dense population of phosphate groups allows for the avid binding of the CPP to divalent metals such as calcium, zinc, copper, manganese and iron (*2*). Due to this unique metal-chelating property, extensive research on CPP has focused mainly on its role in enhancing solubility and hence bioavailability of calcium during milk or casein meal digestion (*3-9*). However, recently the ability of CPP to form organophosphate salts with trace elements has founded new interesting applications as dietary supplements (*10*), medicine and even reinforcement of dental plaques (*11*).

The term CPP was first introduced by Mellander over 50 years ago as a description for the phosphorylated casein hydrolysis products that effectively increased bone calcification, independent of vitamin D, in rachitic infants (*12*). CPP was shown to inhibit precipitation of calcium phosphate *in vitro* (*13*) and subsequently enhanced the deposition of radioactive calcium tracer into femora of rats (*14*). Since the distal intestine is the location where the passive transport of calcium is dominant, an enhanced solubility would indirectly lead to a greater calcium absorption from the intestinal lumen. Earlier research had reconfirmed the effectiveness of CPP in enhancing intestinal solubility but failed to find an increase in femoral deposition of ^{45}Ca, bone calcium mineralization or bone strength in rats fed casein or soy protein diets (*15-18*).

CPP are formed not only through *in vitro* enzymatic proteolysis but they also exist in the intestinal digests of humans, rats and minipigs following ingestion of casein or CPP diets (*19-21*). Since CPP are relatively resistant to further proteolytic breakdown by digestive enzymes shown by their presence in the jejunum, colon and even feces of rats (*22,23*), they carry a great potential as bioactive peptides *in vivo*, especially in the distal sections of the small intestine. Calcium binding capacity does vary among the different phosphopeptide fractions (*24,25*).

This particular and unique chelating property of CPP may potentially be an aid in protecting food- or bio-systems from oxidative damage by removing transition metals responsible for catalyzing lipid peroxidation. Yet, to date, there is no information available demonstrating a potential antioxidant role for CPP. This contribution thus represents an evaluation of the bioactivity of bovine milk caseinophosphopeptides as antioxidants *in vitro*.

Material and Methods

Caseinophosphopeptides (CPP) were obtained from Meiji Seika Kaisha Ltd. (Kawasaki, Japan). CPP-I and CPP-III were commercially produced by treating a whole casein solution [15% (w/v), pH 7.0] with trypsin (Novo Industri, Tokyo, Japan) at 60°C for 2 h to afford the hydrolyzates. The amounts of enzymes for CPP-I and CPP-III production were 0.002% (w/w) and 0.01% of the substrate, respectively. CPP-I was obtained from spray-drying the whole hydrolyzate. CPP-III enriched with phosphopeptides was produced by filtering the insoluble mass from the hydrolysate at pH 4.5 before precipitating the phosphopeptides by adding calcium chloride [1.1% (w/v)] and ethanol [50% (v/v)].

Ferrous chloride, calcium chloride, L-ascorbic acid, 2-deoxy-D-ribose, 2-thiobarbituric acid (TBA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), L-α-lecithin (from soybean), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (diammonium salt, ABTS), Chelax-100 ion-exchange resin, casein and tricine (N-tris [hydroxymethyl] methyl glycine) were obtained from Sigma Chemical Co. (St. Louis, MO). Ferrous sulfate, EDTA (ethylenediminetetraacetic acid), hydrogen peroxide (30%) and trichoroacetic acid (TCA) were from Fisher Scientific (Fairlawn, NJ). Methanol, acetic acid and hydrochloric acid were from Fisher Scientific (Napean, ON). Ferric chloride was purchased from Mallinckrodt (Paris, KY). Sodium hydroxide, potassium dihydrogen orthophosphate and sodium chloride were obtained from BDH Inc. (Toronto, ON). 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemicals USA Inc. (Richmond, VA). Sodium dodecyl sulfate (SDS), ammonium persulfate, glycine, Coomassie blue G-250, Tris base, 2-mercaptoethanol, glycerol and bromophenol blue were from Bio-Rad Laboratories (Richmond, CA). Phosphate buffer was made with distilled deionized water and was eluted through a Chelax-100 column to eliminate the occurrence of transition metals.

Molecular Weight Characterization of Caseinophosphopeptides

Casein, CPP-I and CPP-III were analyzed by SDS-PAGE according to the method of Laemmli (*26*) using 15 %T polyacrylamide separating gel and 4% stacking gel containing 0.1% SDS on the Mini-Protean I Mini-Cell slab gel electrophoresis unit (BIO-RAD Laboratories, Richmond, CA). Protein samples (40μg) were heated at 100°C for 5 min in 0.5M Tris-glycine buffer, pH 6.8 containing 10% SDS. Electrophoresis was carried out at a constant voltage of 100 volts for 1.5 h with a Tris-glycine running buffer. Gels were placed in 0.1% Coomassie blue G-250 staining solution (40% methanol and 10% acetic acid) for 30 min and destained in 40% methanol and 10% acetic acid for 3 x 15 min destain washes.

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Antioxidant Activity of CPP in a Deoxyribose Model

A Fenton reaction model containing 0.1 mM of Fe^{3+} as the catalytic metal was used as described by Halliwell *et al.* (*27*). The substrate (3.6 mM) 2-deoxyribose was mixed together with CPP (0.05, 0.10, 0.50, 1.00mg/ml), 0.1 mM EDTA, 0.1 mM ferric chloride, 0.1 mM ascorbic acid and 1mM H_2O_2 and incubated at 37°C for 1 h. Following incubation, 1ml of 10% (v/v) trichloroacetic acid (TCA) and 1% 2-thiobarbituric acid (TBA) were added and the mixture was boiled for 15 min at 95°C. Absorbance at 532nm was recorded after cooling. The extent of deoxyribose degradation by hydroxyl radicals generated by the Fenton reaction was calculated using the equation:

$$\% \text{ Inhibition} = \frac{\text{A@532nm}_{\text{control}} - \text{A@532nm}_{\text{sample}}}{\text{A@532nm}_{\text{control}}} \times 100$$

Free Radical Scavenging Activity

Scavenging effect of CPP for a stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was measured according to Blois (*28*). CPP (0.05, 0.10, 0.50, 1.00mg/mL) was mixed with 0.1 mM DPPH radical in ethanol prior to an incubation period of 20 min at room temperature. Discoloration was monitored by measuring the absorbance at 519 nm following incubation.

The ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation model was also used to evaluate the free radical scavenging effect of CPP (*29*). Ethanol was replaced with distilled deionized water in the radical preparation. Discoloration was determined by comparing the absorbance at 734nm of the treatment groups with the control after an 8-min incubation at room temperature.

The inhibitory percentage of DPPH and ABTS was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{\text{A@519/734nm}_{\text{control}} - \text{A@519/734nm}_{\text{sample}}}{\text{A@519/734nm}_{\text{control}}} \times 100$$

Statistical Analysis

All data were collected in triplicates and analysed by one way ANOVA ($\alpha \leq 0.05$) followed by a multiple range Tukey post test with the GraphPad Prism Analysis software (GraphPad Software Inc., San Diego CA) to identify significant different among treatment means ($P \leq 0.05$).

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Results and Discussion

Molecular Characterization of Caseinophosphopeptides

Caseinophosphopeptides (CPP-I and -III) were analyzed using SDS-PAGE electrophoresis to determine the approximate molecular weight and purity of samples (Figure 1). Occurrence of bands for the casein sample was consistent with the reported molecular weights of α_{s1}, α_{s2}, β and κ being around 22-23kDa, 25kDa, 23-34kDa and 19kDa, respectively (*30*). As for the CPP-I and CPP-III samples, peptides were distinctively of lower molecular weight with a high concentration of peptides having lower than 6kDa. However, this method was not precise enough to pinpoint the exact molecular weights of the peptides which were reported to have 40% α_{s1}-CN(43-79) and 36% β-CN(1-25) of 4.6kDa and 3.1kDa, respectively (*26*). The phosphopeptides were shown to have a higher purity in the CPP-III sample as compared to the CPP-I sample reflecting the more sophisticated preparation techniques.

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Effect of CPP on Degradation of Deoxyribose

Two different assays were used to investigate the affinity of CPP to protect against hydroxyl radical-induced degradation of deoxyribose in an ascorbic acid mediated Fenton reaction. This assay may also be used to determine whether CPP has the affinity to bind Fe II or III when EDTA is present and also to scavenge hydroxyl radicals in the absence of EDTA. In the *non-site specific binding* deoxyribose assay, the lowering of absorbance values at 532nm indicated that CPP was an effective competitor of EDTA for Fe. However, significant inhibition of deoxyribose degradation was observed only for higher concentrations, 500 and 1000ppm, of CPPI and III. When phosphate buffer substituted EDTA in the *site specific binding* deoxyribose assay, CPP effectively lowered the degradation of deoxyribose by directly competing with the sugar for hydroxyl radicals. Significant inhibition was observed for all 4 concentrations of CPP-I and -III (Table I). Overall, CPP played a role in antioxidation by either rendering the metal catalysts of oxidation unavailable through chelation or by scavenging the hydroxyl radicals generated during the reaction or both.

Effect of CPP on scavenging stable radicals DPPH and ABTS

The extent of discoloration of DPPH radicals was measured by reduction in absorbance values at 517nm. No significant antioxidant activity was observed using this assay. This result could possibly be explained by the solvent ethanol

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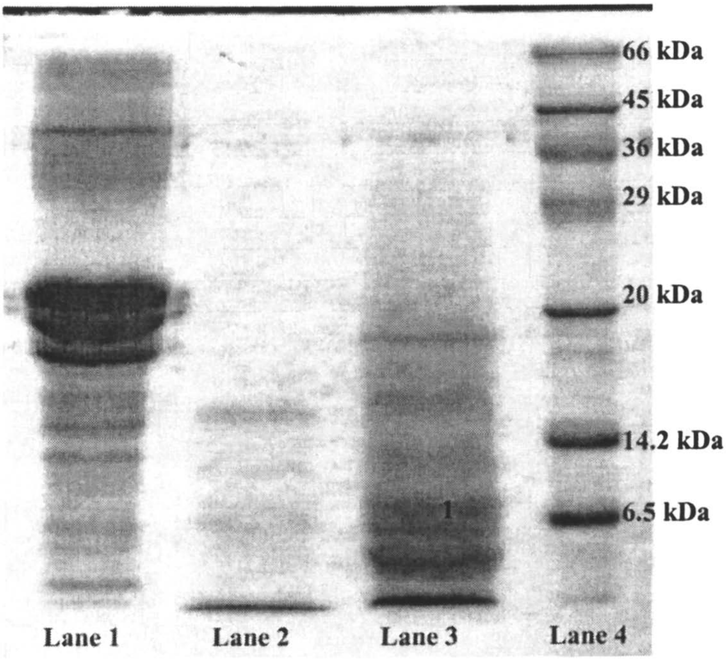
66 kDa
45 kDa
36 kDa
29 kDa
20 kDa
14.2 kDa
6.5 kDa
1
Lane 1
Lane 2
Lane 3
Lane 4

Figure 1. SDS PAGE Gel (15%T) using the Laemmli buffer system (0.375 M Tris, pH 8.8). Lane 1: 40 μg Casein, Lane 2: 40 μg CPPIII, Lane 3: 40 μg CPPI, Lane 4: Molecular Weight Marker.

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Table I. Effect of CPP I and III on % Inhibition[1] of Degradation of Deoxyribose in an ascorbic acid mediated Fenton Reaction in the presence (Non Site Specific Binding) and absence (Site Specific Binding) of EDTA.

		Concentration of CPP (mg/ml)			
Assay	*CPP*	*0.05*	*0.10*	*0.50*	*1.00*
Site Specific Binding	CI	33.6 ± 1.7^{ax}	34.2 ± 3.3^{ax}	42.2 ± 1.5^{abx}	46.2 ± 1.8^{bx}
	CIII	27.1 ± 0.9^{ax}	32.8 ± 0.8^{abx}	40.5 ± 1.4^{bcx}	45.5 ± 0.1^{cx}
Non Site Specific Binding	CI	-0.1 ± 0.1^{ax}	-0.2 ± 0.1^{ax}	18.4 ± 3.1^{bx}	43.7 ± 1.7^{cx}
	CIII	-0.5 ± 0.1^{ax}	-0.5 ± 0.1^{ax}	3.6 ± 1.1^{ay}	25.5 ± 0.4^{by}

[1] % Inhibition = $$\frac{A532nm_{control} - A532nm_{sample}}{A532nm_{control}} \times 100$$

[2] Results are expressed as Mean ± SD, n = 3

[3] Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently (p<0.01)

[4] Means within the same column that do not share a common superscript letter (x and y) are significantly differently (p<0.01)

used. CPP was less soluble in ethanol than water, reducing the extent of contact between the radical and the peptides. Secondly, ethanol may have caused a conformation change in the peptides which rendered the reactive phosphate groups ineffective (Table II).

As an extension of this assay, a free radical that is more hydrophilic in nature was selected. Similarly, discoloration of the ABTS radical, measured at 734nm, occurs when the odd electron on the radical is paired off. CPP-I seemed to be a better electron or proton donor, thus reducing the absorbance of the radical at 100, 500 and 1000ppm. However, significant discoloration was observed for CPP-III only at 1000ppm (Table III). Being able to pair off the unpaired electron on radicals is critical in the termination of oxidation and hence a desired property for antioxidants, CPP's ability to interact with ABTS further suggests that it has antioxidative properties.

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Conclusion

Caseinophosphopeptides (CPP) derived from tryptic digests of bovine casein, molecular weight 19 – 25kDa, were characterized by a molecular weight of less than 6kDa. Two different CPP fractions, CPP-I and CPP-III, were screened for *in vitro* antioxidant activity. CPP-I and CPP-III exhibited significant ($p<0.01$) inhibition of both site-specific and non site-specific degradation of deoxyribose in a Fenton reaction oxidation test. CPP-I was more effective in quenching ABTS radicals than CPP-III. These results demonstrate a potential affinity for CPP to reduce intestinal oxidative stress by reducing exposure of intestinal cells to free radicals.

Acknowlegments

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Table II. Effect of CPP I and III on Scavenging Stable DPPH Radical (expressed as % scavenging[1])

	Concentration of CPP (mg/ml)			
CPP	*0.05*	*0.10*	*0.50*	*1.00*
CI	-5.94 ± 4.8 ^{ax}	-6.43 ± 2.28 ^{ax}	1.24 ± 1.20 ^{ax}	1.24 ± 4.60 ^{ax}
CIII	-7.10 ± 2.12 ^{ax}	-4.87 ± 1.08 ^{ax}	-3.05 ± 1.02 ^{ax}	1.32 ± 0.08 ^{ax}

[1] % Scavenging = $\frac{A519nm_{control} - A519nm_{sample}}{A519nm_{control}}$ x100

[2] Results are expressed as Mean ± SD, n = 3

[3] Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently ($p<0.01$)

[4] Means within the same column that do not share a common superscript letter (x and y) are significantly differently ($p<0.01$)

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Table III. Effect of CPP I and III on Scavenging Stable ABTS Radical (expressed as % scavenging^1 and estimated Trolox equivalence^4)

	Concentration of CPP (mg/ml)			
CPP	*0.05*	*0.10*	*0.50*	*1.00*
CI	1.5 ± 0.7^{ax} (0.5)	7.9 ± 1.0^{by} (2.2)	75.1± 1.6^{cy} (19.4)	91.8 ± 0.5^{dy} (23.7)
CIII	0.2 ± 1.0^{ax} (0.2)	1.1 ± 0.8^{ax} (0.4)	3.2 ± 0.7^{ax} (1.0)	8.4 ± 0.8^{cx} (2.3)

[1] % Scavenging = $\dfrac{\text{A734nm}_{\text{control}} - \text{A734nm}_{\text{sample}}}{\text{A734nm}_{\text{control}}} \times 100$

[2] Results are expressed as Mean ± SD, n = 3

[3] Means within the same row that do not share a common superscript letter (a, b, c and d) are significantly differently ($p<0.01$)

[4] Means within the same column that do not share a common superscript letter (x and y) are significantly differently ($p<0.01$)

[5] Values in parentheses denote the estimated Trolox equivalence (μM) calculated using the standard curve equation $y = 3.896x - 0.5844$ where x stands for micromolars of Trolox and y for % scavenging.

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Chapter 22

Structural Changes of Soymilk Proteins during Heating and Cooling, and Freeze-Gelation of Soymilk

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The heated soymilk could be converted to a gel-like coagulate by freezing. In this freeze-gelation, cooling of heated soymilk at -5°C before freezing (precooling) was very important. The structural changes of soymilk proteins and interactions among them during heating and cooling was thus evaluated. Surface SH contents and surface hydrophobicities of soymik proteins, which increased by heating of soymilk, decreased to a lesser extent by cooling at -5°C than by cooling at room temperature. Precooling was shown to suppress refolding of thermally denatured proteins. Further, considering the effects of some additives such as sodium dodecylsulfate, 2-mercaptoethanol and sucrose, the inteaction among proteins and the freeze-gelation mechanism were discussed.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

A large number of people drink soymilk for its health benefits, especially in Japan, where its consumption has gradually increased over the past several years. Soymilk is an emulsion consisting mainly of soybean proteins and lipids, which nutritionally and functionally have excellent qualities. It is utilized as not only beverage itself, but also, for its ingredients. Asians including Japanese have been consuming tofu, soybean curd, yuba, and sheets of dried soymilk skin among others. As for the gel derived from soy protein, heat induced gel (*1,2*) and tofu gel (*3-6*) are well characterized, and there are too many reports in the literature during the past half century. Further, the soy protein dispersion or paste have been reported to form gel-like coagulates by heating and freezing (*7-9*), or heating and refrigeration (*10*). Here soymilk was used as a sample instead of soy protein dispersion; it was converted to gel-like coagulate by heating and freezing (*11*). Raw soymilk was heated for 3 min in an autoclave, then immediately precooled in a freezer at -5°C for 2 h. The cooled soymilk was put into a freezer at -20°C and stored for 14 days. The frozen soymilk was thawed in a warm water bath. Soymilk freeze-gel is similar to soft tofu or yogurt (Figure 1). This freeze-gel is processed without the addition of coagulants such as calcium sulfate, magnesium chloride, glucono-δ-lactone, etc., thus differing from traditional tofu gel (*3-6*). The significance of heating and cooling steps of soymilk was of interest in order to clarify the structural changes of proteins in the soymilk during heating and cooling, including precooling step, in terms of the soymilk freeze-gelation.

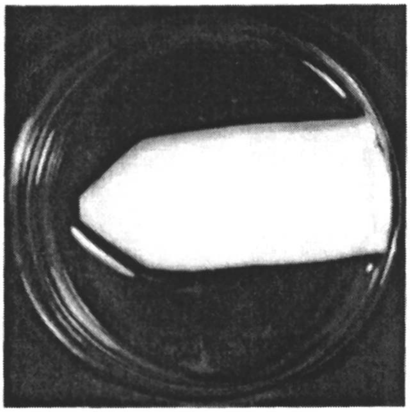

Figure 1. Soymilk freeze-gel

These structural changes of protein molecules are thought to be important for the interactions among the proteins. Interactions among protein molecules in aggregation have been widely investigated. Disulfide, hydrophobic and electrostatic interactions are considered important for heat-induced gelation (*2,12*). In the frozen insolubilization of soybean protein solutions, disulfide bond

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formation and exchange were reported to be important intermolecular interactions (*8*), while for cold gel formation, the hydrophobic interactions were more important (*10*). Thus, the interactions among protein molecules during freeze-gelation, in which the precooling step is considered to be very important for uniform texture of the freeze-gel (*11,13*) was studied. The precooling step allows the formation of spherical ice crystals, which were found to be distributed uniformly in frozen soymilk during freeze-storage (*13*). Attempts were also made to clarify the significance of freeze-storage as affected by pre-cooling in the protein structures of soymilk. For this, the free thiol content and the surface hydrophobicity of protein were estimated during precooling. The effect of precooling on the structure of heated soybean protein during cooling is discussed herein. The freeze-gelation mechanism of soybean, especially, the interactions among the protein molecules, were also studied.

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Structural Changes in Soymilk Proteins During Heating, Cooling and Freezing

Generally, soymilk was prepared by sucessively heating and squeezing soybean which was ground with water. However, we prepared raw soymilk under mild heating condition below 70°C, where main proteins in soybean are considered to maintain their native structures. The raw soymilk and the heated soymilk, prepared by heating of the raw soymilk at 100°C, were frozen in a freezer (-20°C) for 7 days. Appearances of products after thawing in warm water bath (30°C) are shown in Figure 2. The heated soymilk precipitated by freezing and thawing, but the raw soymilk did not. These data suggest that heating process is essential for frozen precipitation of soymilk, namely thermal denaturation of soymilk proteins would lead frozen precipitation of proteins and other constituents. Then the effect of frozen storage time of heated soymilk on the precipitation after freeze-storage was estimated. At the beginning, the precipitation increased with the increase of the frozen time and reached plateau for about 14 days of frozen storage at -20°C. The frozen storage period affected a little the precipitation of soymilk, but precipitation increased with the heating time and reached plateau for 3 min of heating of soymilk (data not shown). Raw soymilk was reported to form freeze-gel by sucessive heating (110°C, 3 min), precooling (-5°C, 2 h) and frozen storage (-20°C, 14 days) (*11*). Behavior of precipitation of soymilk described above is almost consistent with the previously reported data. Further, the precooling was reported to be essential for the formation of freeze-gel (*11*). So, the thermal denaturation behaviors of soymilk proteins during heating and cooling was estimated.

At first, surface SH contents of soymilk proteins were measured using

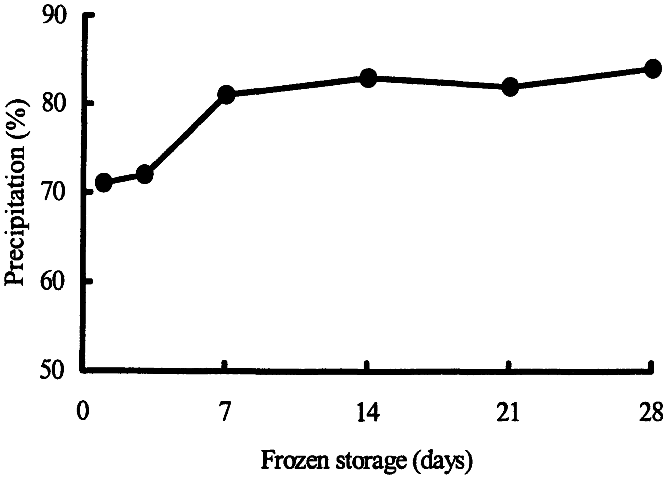

Figure 2. Freezing and thawing treatment of heated or unheated soymilk.

2,2'-dithiobis-(5-nitropyridine) (DTNP) (*14,15*). Soymilk was adequately diluted with a 0.1 M, pH 7.6 phosphate buffer. An aliquot (2 mL) was mixed with 5×10^{-4} M DTNP in ethanol (0.5 mL) and incubated for 20 min at room temperature. The resulting solution was mixed with a 10 % perchloric acid solution (2.5 mL) and centrifuged for 10 min at 1,500 xg. The supernatant was filtered and its absorbance read at 386 nm. Surface SH increased by heating and reached a maximum after 1 min of heating (Figure 3), but then gradually decreased with the increase of heating time. These data show that thermal denaturation increased surface SH groups by breakikng S-S bonds and exposing interior SH groups by unfolding of the protein molecules. Further heat treatment may accelerate the exchange and reformation of S-S bond and decomposition of SH group to H_2S or other compounds, and result in a decrease of the surface SH. Therefore, the SH content decreased to about two third of the value immediately after heating by cooling at room temperature for 2 h. However, it maintained almost equal level to that after heating, when it was left at -5°C. These data show that rapid cooling of soymilk inhibits reforming of S-S bonds and/or coverings of the SH groups by refolding of the protein molecules. These suggest that the cooling of the heated soymilk at -5°C maintains the surface SH, namely potent reactivity by the S-S formation to be higher.

The surface hydrophobicities of the proteins in soymilk was subsequently evaluated. The surface hydrophobicity of protein was measured using 1-(anilino) naphthalene-8-sulfonate (ANS) (*16*). A properly diluted sample (0.1 mL) was

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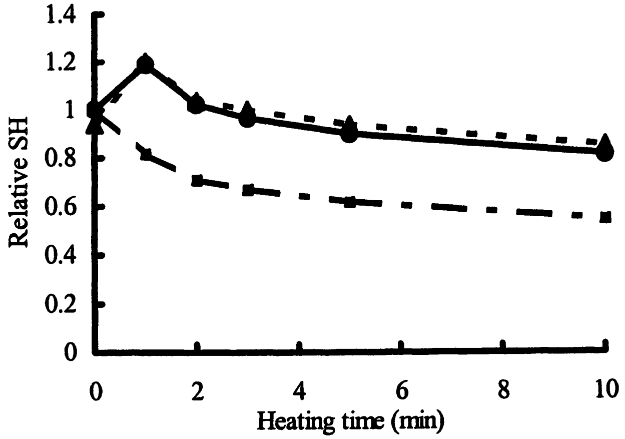

Figure 3. Surface SH of soymilk protein after heating and cooling

mixed with 0.01 M phosphate buffer (pH 7.0, 4 mL) and 8×10^{-3} M ANS solution (0.02 mL). The mixture was excited at 390 nm and the relative fluorescence intensity was measured at 470 nm in a fluorescence spectrophotometer (F-2000, Hitachi, Ltd., Tokyo, Japan). As a result (Figure 4), the surface hydrophobicity of soymilk increased by heating, however the value was almost maintained from 0.5 to 3 min of heating. Unfolding of protein molecule is genarally considered to allow interior hydrophobic region of the protein to

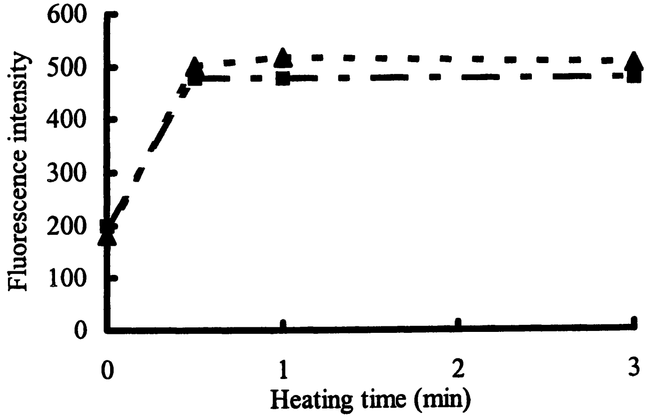

Figure 4. Surface hydrophobicity of soymilk protein after heating and cooling (Reproduced from reference 17)

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expose outside, so increase of surface hydrophobicity was responsible for partial unfolding of soymilk proteins. The hydrophobicity was slightly but significantly higher by cooling at -5°C than at room temperature. Because of the lipids and other constituents, the measurements of hydrophobicity in soymilk may contain some inaccuracies. Even given these restrictions, the data coincided well with the result from surface hydrophobicity in APP solution (data not shown). The surface hydrophobicity, which increased with heat treatment, might maintain at a relatively higher level by rapid cooling to the supercooling state.

The precooling of heated soymilk at -5°C suppressed the decrease in surface thiol content and surface hydrophobicity of soybean proteins, which had increased during the heating step. This result might also show that precooling suppressed the refolding of the protein molecules, which had been partially destroyed or unfolded by heat treatment. High levels of surface thiol and surface hydrophobic areas were required to form three-dimensional networks during frozen storage. In earlier work (*13*), the significance of the precooling step was reported to form uniform, fine ice crystals. The high level retention of surface thiol and hydrophobicity is considered to be another significant aspect of the precooling for freeze-gelation.

In order to evaluate the secondary structures of protein molecules during heating and precooling of soymilk. Fourier transform - infrared spectra (FT-IR spectra) were recorded by a System2000 (Perkin Elmer Co.) using Horizontal ATR method. As a result (Figure 5), IR spectra of soymilk showed the amide band I which is composed of the absorptions derived from amide bonds (-CO-

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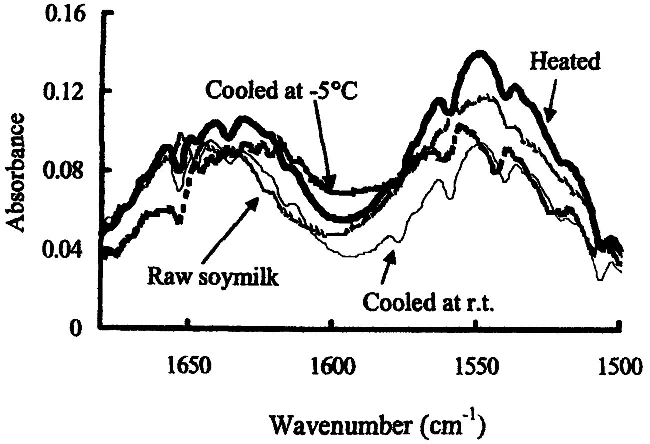

Figure 5. IR spectra of soymilk after heating and cooling

NH-) and show the proportions of secondary structures, namely α-helix, β-sheet, β-turn and non- ordered structure. The amide band I of raw soymilk had a peak at about 1650 cm^{-1} which shifted in heated soymilk to low frequency (~1630 cm^{-1}), showed the increase of β-sheet-like or extended structures (*18*) by heat denaturation. These data may show that soymilk protein molecules were partially and slightly unfolded by heat treatment although they generally maintained their steric structures. During cooling of soymilk at room temperature, the amide band I was almost back to the native state, so the protein moelcules were considered to be almost refolded. However, the shifted band was maintained during cooling at -5°C. Heat denatured proteins were refolded during cooling, but cannot refold by cooling at -5°C because rapid cooling decreased the velocity of conformational changes of proteins.

From these data, soymilk proteins were partially denatured by heating at 110°C, changed their secondary structures and exposed interior SHs and hydrophobic regions, and resulted in an increase of the reactivity to other molecules. Further, cooling of thermally denatured protein lead to partial refolding but rapid cooling (precooling at -5°C) caused slow refolding to be slower and maintained the reactivity between protein molecules at relatively high levels.

Interactions Among Protein Molecules in Soymilk Freeze-gel and Freeze-gelation Mechanism

In order to estimate the involvement of S-S bonds, hydrophobic interactions and hydrogen bonds, the effects of some additives such as sodium sulfite, sodium dodecylsulfate (SDS) and sucrose on the formation of soymilk freeze-gel were evaluated. Figure 6 shows the effect of sodium sulfite which reduces the intra- and intermolecular S-S bonds of protein molecules. Raw soymilk was mixed with sodium sulfite and then heated, precooled and frozen (*11*). After 2 weeks, the frozen samples were thawed in water at 30°C. In comparison with the control sample (Figure 1), the addition of 0.1% sodium sulfite partially suppressed gelation and led to a heterogeneous precipitation (Figure 6a). Increased sodium sulfite (0.5%) completely inhibited coagulation of soymilk (Figure 6b). These data are cosistent with those reported by Hashizume (*9*), indicating that the formation of intermolecular disulfide bonds was important for the insolubilization of soybean protein during frozen storage. Hydrophobic interactions were also considered to be important among protein molecules. The addition of SDS led to a partially collapsed surface on the freeze-gel at 0.1% (Figure 7a) and to heterogeneous precipitation at 0.5% (Figure 7b).

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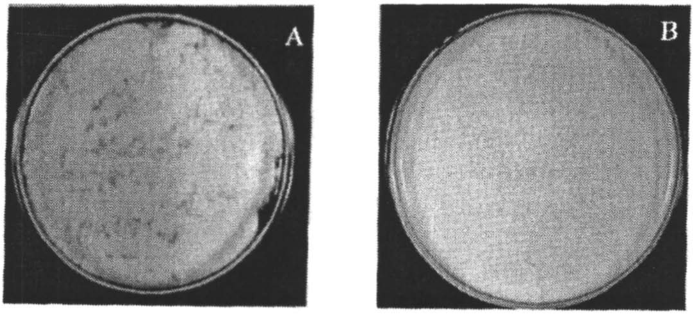

Figure 6. Effect of sodium sulfite on freeze-gelation of soymilk (Reproduced from reference 17. Copyright 2000 American Chemical Society.)

A, 0.1% of sodium sulfite added; B, 0.5% added.

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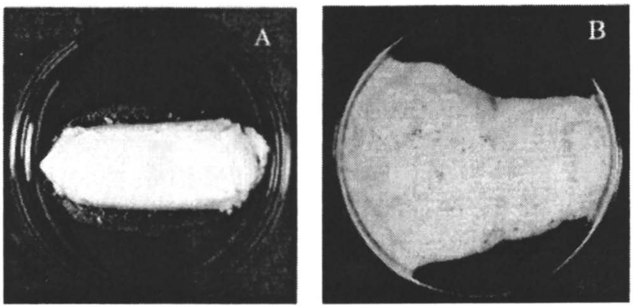

Figure 7. Effect of sodium dodecylsulfate (SDS) on freeze-gelation of soymilk (Reproduced from reference 17. Copyright 2000 American Chemical Society.)

A, 0.1% of SDS added; B, 0.5% added

Apparently, a greater concentration of SDS than sodium sulfite was needed to inhibit the formation of the freeze-gel. It is considered that soybean protein subunits, which were dissociated during the heating step (*2*), were associated with other subunit through intermolecular disulfide bonds or hydrophobic interactions. The resulting aggregates then interacted with other aggregates to form insoluble coagulates. Differing from the above data, the addition of a small amount of sucrose allowed the freeze-gel to be stabler and smoother (data not shown). However, further addition of sucrose lowered the stability of the gel.

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This unique effect of sucrose may show that sucrose affects not only hydrogen bonds among proteins, but also hydrophobic interactions. The above results showed that disulfide, hydrophobic and hydrophilic interactions are important in the formation of gel texture.

Proteins in the soymilk were partially denatured and unfolded by heat treatment. In this state, some of the SH groups and hydrophobic regions were considered to be exposed on the surface of the protein molecules. Then, if the heated soymilk was left at room temperature, the denatured proteins were partially refolded and surface reactivity, which included SH and S-S exchanges and hydrophobic and hydrophilic interactions, lowered. However, if the soymilk was put into a freezer controlled at -5°C, the refolding and S-S reformation of the denatured proteins were delayed and the surface reactivity retained at a relatively high level. When the precooled soymilk was frozen, the protein molecules were freeze-concentrated by forming ice crystals and closely interacted with one another through S-S and hydrogen bonds and hydrophobic interactions. During the frozen storage, interactions among proteins were strengthened and allowed the gel to be more stable.

Sucrose showed some different effects on the freeze-gelation, and the effect depended on the amount added to soymilk. Gekko *et al.* (*19*) reported that polyols like sorbitol stabilized the protein structure but destabilized the gel structure. The same authors speculated that the thermal stabilization of proteins can be responsible for the strengthening of intramolecular hydrophobic interaction. A small amount of sucrose addition enhanced the hydrophobic interactions among protein molecules resulting in stable gel formation by improving the association or entanglement of the peptide chains. However, a large amount of sucrose was considered to enhance hydrophilic interactions between proteins and water as well as the hydrophobic interactions, and these interaction to form destabilized cross-links through the hydrogen bonds among proteins.

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Chapter 23

Amino Acids and Volatile Compounds in the Fermentation of Inoculated Musts: Biogenic Amines in the Wines

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The evolution of amino acids and volatile compounds during fermentation of garnacha must inoculated with three strains of *S.cerevisiae* was determined. The concentration of biogenic amines in the resultant rosé wines were evaluated and the results compared with those obtained from the same garnacha must fermented with indigenous yeasts. The inoculated strains had an influence on the utilization of amino acids in the second half of fermentation only. In terms of volatiles, the inoculated strains in no case produced wines with a higher quantity of esters and higher alcohols than the wine from the must fermented by the indigenous yeasts. The evolution of the esters during the fermentation was different in the inoculated samples than in the control sample, something which did not occur with the higher alcohols. The concentration of biogenic amines in the wines was slightly different depending on the inoculated strain, although in no case did they show concentrations sufficient to cause toxic problems for the consumer.

In Nutraceutical Beverages; Shahidi, F., et al.;
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There has been considerable controversy about the use of selected pure strains in wine fermentation. For that reason it is important to determine the influence of this vinification technique, in the evolution of fermentation and in the composition of wine. Several groups have studied the nitrogen needs of different yeasts in synthetic media (*1-3*), however, these needs in musts used for vinification require attention. Although part of the wine aroma comes from the grape, the components that make up the main part of the aroma develop during fermentation (*4*). In the inoculated musts the wild yeasts make an important contribution to the wine aroma, however, the inoculated strains have a decisive influence in the volatile fraction of wine when it predominates in the fermentation (*5*). The concentration of biogenic amines in wines depends on various factors, among which the yeast strain that intervenes in the fermentation stands out (*6*). These compounds could have a negative effect on the aroma of the wine and moreover, some have a toxic action.

In this investigation a garnacha must was inoculated with three selected yeast strains of *S. cerevisiae* in order to study the influence of yeast strain on: a) the utilization of amino acids during fermentation; b) the evolution of volatile compounds during fermentation and their concentrations in the wines, and c) the concentration of biogenic amines in the wines. An uninoculated garnacha must was used as the control sample.

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Experimental

Samples and Vinification

The must used was *Vitis vinifera* var. *garnacha*. The must was inoculated with active dry *S. cerevisiae* var. *cerevisiae* yeast; the strains used were Na33, D47 and K1M. Na33 strain was isolated in musts from the region of Navarra (North of Spain) and it has neutral phenotype. D47 and K1M strains have K2 killer phenotype. The former yeast comes from the French region of Côtes du Rhône and the latter comes from the French region of Languedoc. The must was kept in contact with the skins between 17 and 20 h and the fermentation was made at a controlled temperature of 18±2°C. Both the inoculated fermentations as well as the spontaneous one (control sample) were carried out in duplicate, using modular bioreactors of 5 L. The organoleptic characteristics of the wines obtained were evaluated by experts with wide knowledge of the rosé wines produced in Navarra. Sensory analysis showed that the wines obtained from inoculated fermentations did not possess a higher organoleptic quality than the control wine, from which a better aroma was detected.

Preparation of Sample and HPLC Analysis of Free Amino Acids

Analysis was performed with a Waters high performance liquid chromatograph (Waters Chromatography Division, Milford, MA) equipped with two 510 pumps, a 717 Plus Autosampler, and a 486 UV-vis detector used at 254 nm. The Pico· Tag method used is described by Ancín *et al.* (*7*). The column used was a Pico· Tag reverse phase (300 mm x 3.9 m id) with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica (Waters Chromatography, Milford, MA).

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Analysis of Volatile Compounds by Gas Chromatography

It was felt necessary to use two methods of analysis because the volatile compounds of the wine have different volatilities and they are found in a very wide range of concentrations. Instrumentation as well as analytical and preparative conditions have been described already (*8*). The compounds of high volatility and concentration were analyzed by direct injection of 0.5 µL of sample in a Shimadzu GC-R1A gas chromatograph (Shimadzu Kyoto, Japan) with a Flame Ionization Detector. The column used (4 m x 3.2 mm i.d.) contained Carbowax 1500 (15%, w/w) on Chromosorb WHP as stationary phase (Teknokroma, Barcelona, Spain). The compounds of the middle-range volatility and, in general, present in lesser concentrations than the former ones, were previously extracted, concentrated, and then analyzed by GC-MS. For these analysis a Hewlett-Packard 5890 (Palo Alto, CA) was used, equipped with a mass spectrometry detector HP 5971 A, and an automatic injector HP 7673. The chromatographic separations were performed with a capillary column TR-WAX (30 m x 0.32 mm i.d., 0.25 µm film thickness; Teknokroma, Barcelona, Spain) of polyethyleneglycol, cross-linked and chemically bonded (Teknokroma, Barcelona, Spain).

Preparation of Sample for HPLC Analysis of Biogenic Amines

The method used was based on that described by Busto *et al.* (*9*). The sample was derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Analyses of the derivatized amines were performed with a Waters high performance liquid chromatograph, with a 474 Fluorescence detector, using 250 and 395 nm as the excitation and emision wavelengths respectively. A reverse phase column (300 mm x 3.9 mm i.d.) with a dimethyloctadecylsilyl bonded to amorphous silica (Waters Chromatography Division, Milford, MA) was used, with a stationary phase of dimethyloctadecylsilyl bonded to amorphous silica (Waters Chromatography Div., Milford, MA, USA).

Technique of Polymerase Chain Reaction (PCR)

The predominance of the inoculated yeasts over the indigenous population was confirmed through this technique. The analyses were made in the Sigmo laboratory of Nantes (France), following the method of Lavallée *et al.* (*10*).

Results and Discussion

Evolution of Free Amino Acids during Fermentation

Figure 1 shows the utilization of the most abundant free amino acids from the garnacha must (glutamic acid, aspartic acid, alanine, threonine, γ-aminobutiric acid, arginine and proline) during the first half of fermentation. It can be seen that these amino acids, except proline, were consumed in a similar way in all the samples, independent of the yeast strain used. The high consumption of arginine stood out. This amino acid was the principal source of nitrogen for the yeasts in all the samples since, as well as being the most consumed one, it has four atoms of nitrogen per molecule. Proline followed a different tendency compared to the other amino acids and it was excreted in all cases. The liberation of proline may be due to the degradation of arginine (*11*).

During the second half of fermentation differences in the utilization of the amino acids among the samples began to be appreciated (Figure 2). Thus, in the musts inoculated with D47 and K1M strains and in the control sample, the amino acids were excreted in variable amounts, although the excretion (except in the case of the proline) was higher in the inoculated musts. Contrary to this tendency, in the sample inoculated with Na33 strain, low amounts of amino acids were consumed (*12*). The excretion which took place in D47, K1M and control samples was due to various causes, among them, the high concentration of ethanol in the medium. This cellular toxic inhibits the transport systems of amino acids in the yeasts (*13*) and favors the passive excretion of these nitrogenous compounds into the medium (*14*). The higher excretion of amino acids in the samples inoculated with D47 and K1M strains could be caused by the sum of the toxic effect of ethanol with the action of the killer toxin secreted by these two strains. The killer toxin inhibits the transport of amino acids and favors the excretion of these compounds to the medium by the formation of porous in the plasmatic membrane of the sensitive yeasts (*15, 16*). In the case of the sample inoculated with the Na33 strain, the consumption of amino acids up to advanced stages in the fermentation would indicate that this strain shows a high tolerance to the toxic effects of ethanol.

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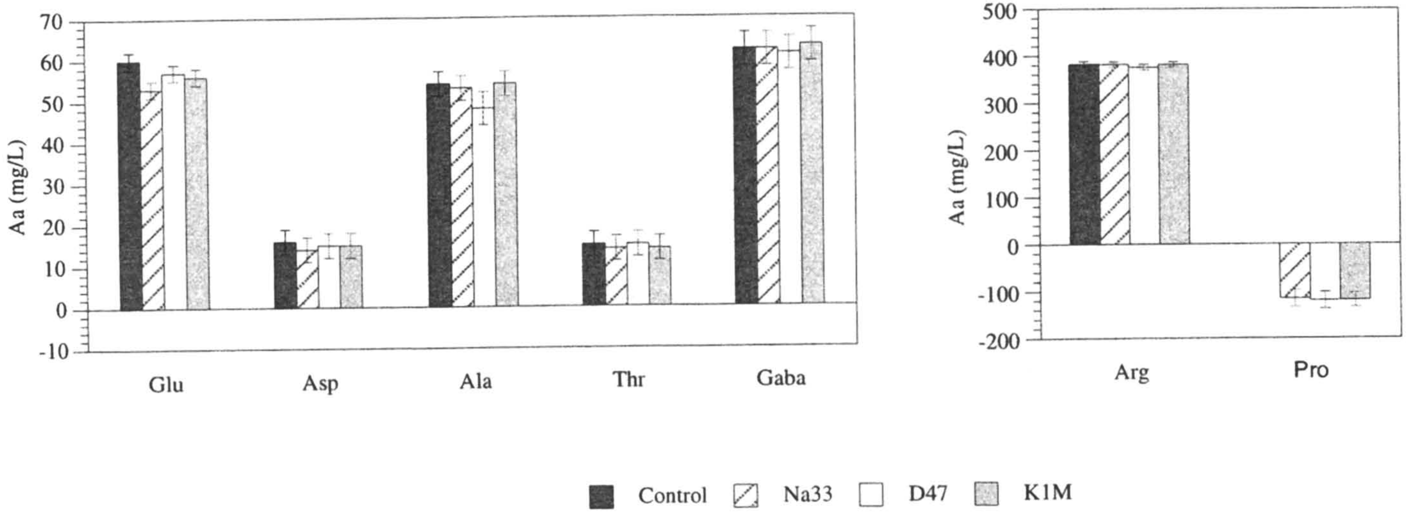

Figure 1. Utilization of free amino acids during the first half of fermentation (+ consumption, - excretion). The results are mean values of six determinations with standard deviations.

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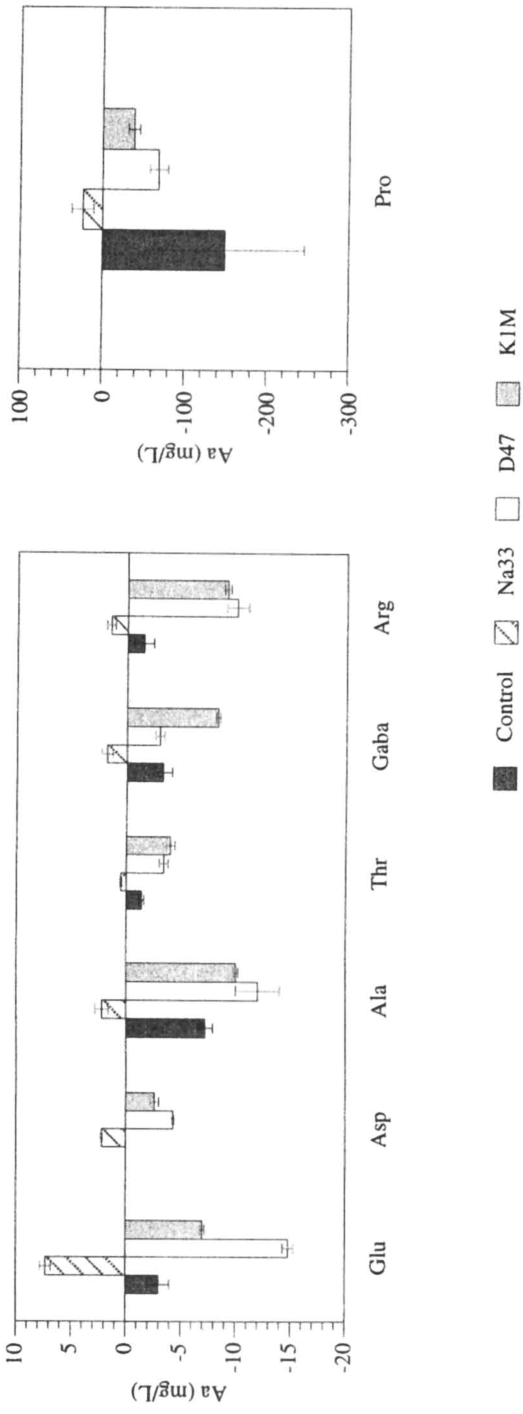

Figure 2. Utilization of free amino acids during the second half of fermentation (+ consumption, - excretion). The results are mean values of six determinations with standard deviations.

Volatile Composition of Rosé Wines: Evolution during Fermentation

Higher Alcohols

According to origin, two groups of wine alcohols can be considered; those that are synthesized from a keto acid resulting from the oxidative deamination of an amino acid or involved as an intermediate in its biosynthesis, and those that are not produced directly from an amino acid, but from a keto acid that takes part as an intermediate in cell glucidic metabolism. The former group includes isoamyl alcohol, isobutyl alcohol, phenylethanol, tyrosol, and tryptofol, which can be synthesized from leucine (and isoleucine), valine, phenylalanine, tyrosine, and tryptophan, respectively (*17*). The sum of n-propanol, isobutyl alcohol, isoamyl alcohol, n-hexanol, 2-phenylethanol, tyrosol and tryptofol in wines from the garnacha musts inoculated with NA33, D47 and K1M yeast strains were respectively 365, 456 and 395 mg/L and in the control sample 442 mg/L (Figure 3a). Therefore the yeast employed was important in the concentration of higher alcohols in the wines (*8*), which coincides with the results of Boulton *et al.* (*18*). The evolution of these substances was similar in all fermentations (Figure 3a). It can be seen that the formation of these alcohols took place in the fermentation, after the majority of the consumption of amino acids had taken place. This apparent contradiction is explained by the fact that the synthesis of these alcohols can also be produced from the excess of their corresponding keto acids (*19*).

Esters

Esters can arise during yeast metabolism either from the alcoholysis of acyl-CoA or from the carbon skeletons of amino acids. Several investigators in the discussion of their results do not include ethyl acetate with the rest of the esters because it makes a different contribution to the aroma of the wine (*20, 21*). Thus, "total esters" have been considered as the sum of the concentrations of isoamyl acetate, 2-phenylethyl acetate, ethyl octanoate, ethyl decanoate, ethyl lactate, and diethyl malate. The concentration of total esters (Figure 3b) in wine control (8.2 mg/L) was higher than in wine fermented by Na33 (4.6 mg/L) and similar to the concentration of esters in the wines from the musts inoculated with D47 (7.5 mg/L) and K1M (8.0 mg/L) strains (*8*). Therefore, the concentration of esters in the wine was different depending on the strain responsable for the fermentation, which coincides with the results of Soles *et al.* (*5*). As can be seen in Figure 3b, the esters were synthesized especially during the second half of fermentation, due to the fact that their formation is inhibited by the presence of oxygen (*22*). In the figure is also observed that the evolution of these compounds during the

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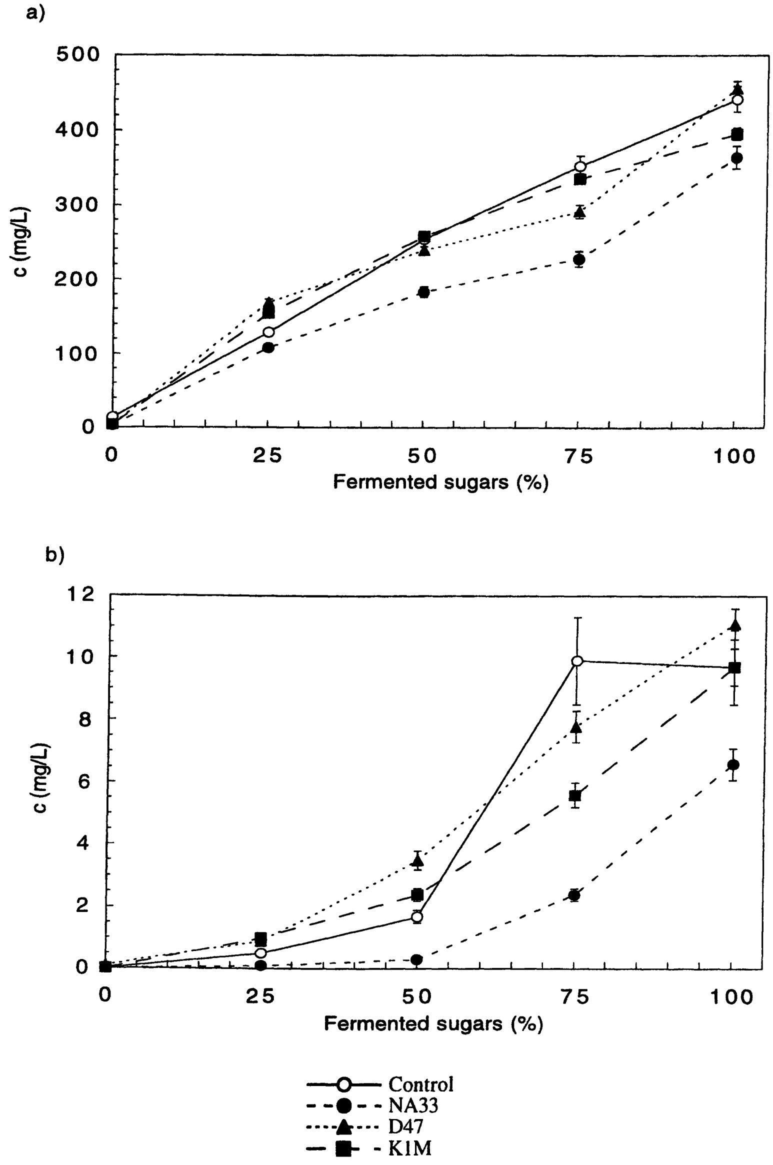

Figure 3. Evolution of the volatile compounds during fermentation a) higher alcohols, b esters. The results are mean values of four determinations with standard deviations.

fermentation was different depending on the strain involved in the fermentation. The control sample reached the maximun concentration at 75% of consumed sugars, while in D47, K1M and Na33 samples, the synthesis was increasing up to the end of fermentation. These results can be explained in accordance with studies of Mauricio *et al.* (*23*), who found distinct activities of the enzyme alcohol acetyltransferase (ATT), and of the cellular hydrolytic esterases depending on the strain of *S. cerevisiae.* In the control fermentation, it seems that a greater activity of AAT existed, but also of the cellular hydrolytic esterases which work at the end of fermentation. Inoculated yeasts showed less synthetic activity, but also less hydrolytic activity.

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Content of amines in the wines

The amines analyzed were putrescine, spermine, phenethylamine +spermidine, histamine, tyramine, dimethylamine, ethylamine, isopropylamine, pyrrolidine, cadaverine, amylamine, hexylamine, diethylamine and isobutylamine (*24*). Only the first nine showed higher concentrations than the detection limit of the method, as shown in Table I.

Table I. Concentration of amines in the wines. The results are shown in μg/L and are given with their standard deviation.

	Control	*Na33*	*D47*	*K1M*
Putrescine	11536 ± 523	8751 ± 288	11977 ± 515	10224 ± 865
Spermine	27 ± 3	24 ± 4	92 ± 3	164 ± 8
Phe + Spd [a]	99 ± 8	58 ± 4	234 ± 24	158 ± 15
Histamine	428 ± 34	406 ± 17	436 ± 37	514 ± 31
Tyramine	224 ± 14	227 ± 24	b	101 ± 11
Dimethylamine	279 ± 28	270 ± 11	172 ± 16	276 ± 18
Ethylamine	438 ± 32	419 ± 13	965 ± 39	700 ± 13
Isopropylamine	b	b	81 ± 2	99 ± 4
Pyrrolidine	240 ± 16	170 ± 9	341 ± 16	472 ± 10

[a] phenethylamine and spermidine; [b] not detected

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Putrescine was, by far, the most abundant amine in all wines tested (Table I). This result coincides with that of other authors, who have found that this amine was most abundant both in the must (*25, 26*) and wine (*27-29*). The

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concentration of putrescine was slightly lower in the Na33-aided fermentation than in other wines. Spermine and phenethylamine+spermidine were present in low concentrations in all wines, although slight differences were observed (Table I). It is considered by Soufleros *et al.* (*29*), that wine with 3 mg/L of phenethylamine could provoke negative physiological effects. In our samples, all wines analyzed showed concentrations of phenethylamine lower than this value, since the sum phenethylamine+spermidine never exceeded 0.23 mg/L. Histamine and tyramine were found in low quantities in all wines and there were no appreciable differences in the content of these amines in the samples. All the wines showed much lower concentrations of these amines than those reported by Daeschel (*30*) and Soufleros *et al.* (*29*) so that the wine can have toxic effects if consumed in large quantities (histamine: 8-20 mg/L, tyramine: 25-40 mg/L).

The volatile amines (dimethylamine, ethylamine, isopropylamine and pyrrolidine) were found in all the wines in lower quantities than those needed to produce negative effects in the aroma (*31*). Ethylamine, isopropylamine and pyrrolidine showed somewhat higher concentrations in the wines from the musts fermented with D47 and K1M strains. Dimethylamine showed similar concentrations in all samples except the D47 sample where it was lower.

Conclusions

The type of yeast responsible for the fermentation did not influence the consumption of amino acids at the beginning of the process. However, during the second half of fermentation differences were observed among different samples. Na33 strain consumed amino acids up to the end of the process, while in the samples fermented by the killer yeasts, there was greater excretion of amino acids than in the control, and so these wines were the least stable microbiologically. The inoculated strains in no case produced wines with a higher quantity of volatile esters and higher alcohols than the wine from the must fermented by the indigenous yeasts. The evolution of esters during fermentation was different in the inoculated samples than in the control sample, something which did not occur with the higher alcohols. The concentration of biogenic amines in the wines was slightly different depending on the inoculated strain, although in no case did they show concentrations sufficient to cause toxic problems for the consumer or to cause any alteration of the sensory quality of the wine.

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Chapter 24

Powerful Aromatic Volatile Thiols in Wines Made from Several *Vitis vinifera* Grape Varieties and Their Releasing Mechanism

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By extracting specific volatile thiols using *p*-hydroxymercuribenzoate, 4-mercapto-4-methylpentan-2-one (4MMP), 4-mercapto-4-methylpentan-2-ol (4MMPOH), 3-mercapto-3-methylbutan-1-ol (3MMB), 3-mercaptohexan-1-ol (3MH), 3-mercaptohexyle acetate (3MHA), and furanmethanethiol (2FM) were identified in wines made from several *Vitis vinifera* white and red grape varieties. The assay of volatile thiols in certain wines made from Sauvignon blanc, Gewürztraminer, Riesling, Colombard, Petit Manseng and botrytized Semillon confirmed the contribution of 4MMP, A3MH and 3MH to their characteristic aromas reminiscent of box tree, grapefruit, and passion fruit. 2FM, exhibiting a strong roasted coffee aroma, is present in white and red wines elaborated in new oak barrel at much higher concentrations than the perception threshold. 4MMP, 4MMPOH and 3MH can be released during alcoholic fermentation from their S-cysteine conjugate precursors. 2FM is generated in white wines during barrel fermentation. The formation is due to yeast transformation of furfural released from toasted staves.

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Introduction

Volatile thiols are extremely odoriferous molecules that probably contribute to the aroma of many foods, including fruit and grilled meat (*1*). Their positive contribution to the aroma of wines made from certain grape varieties has now been proven. However, the fact that these complex natural substances are only present in trace amounts makes them difficult to identify and assay. The analytical methods previously available involved tedious, complicated purification phases that were also rather nonspecific.

We concentrated our investigations on the highly characteristic bouquet of Sauvignon blanc wines, with their broad palette of aromas featuring bell peppers, box tree, blackcurrant buds, grapefruit, passion fruit and, in some cases, smoke. This unusual bouquet had long attracted the attention of ampelographers, winemakers, and, of course, aroma chemists. The fact that at least two volatile thiols reminiscent of box tree have been found in the varietal aroma of Sauvignon blanc wines provides a basis for further research into the varietal aromas of non-Muscat grape varieties (*2,3*).

We have spent several years working on the development of a specific method for extracting volatile thiols. We were particularly interested in the property of an organomercuric compound, *p*-hydroxymercuribenzoic acid (*p*-HMB), to form specific combinations with volatile thiols, which break down in the presence of excess quantities of other thiols, such as cysteine or glutathion (*4*). This property was first applied to identify a compound with one of the lowest perception thresholds in Sauvignon blanc wines, 4-mercapto-4-methylpentan-2-one (4MMP), which has a odor reminiscent of box tree and broom (*3*). In the same way, Lavigne *et al.* (*5*) later demonstrated that two other volatile thiols, 2-mercaptoethyl acetate and 3-mercaptopropyl acetate, also contributed to the "roasted meat" aromas found in some Semillon and Sauvignon blanc wines. Improvement in our analytical techniques then made it possible to develop a method for extracting specific volatile thiols (*6,7*). The compounds responsible for the fruity nuances in Sauvignon blanc wines, e.g. passion fruit, grapefruit, etc., had not been identified until we carried out this research. We show that the trace amounts of volatile thiols present in wines contribute to the varietal aroma of certain *Vitis vinifera* grape varieties.

Furthermore, the volatile thiols that have been identified in wines are almost entirely absent from the corresponding must. The development of varietal aroma in wines made from non-Muscat grape varieties, such as Sauvignon blanc, during alcoholic fermentation remained a largely unexplained phenomenon. Indeed,

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although work by Darriet *et al.*, (*8*) had demonstrated the existence of a non-glycosylated precursor of 4MMP in Sauvignon blanc grapes and must, this compound was difficult to purify and had not yet been identified. Its existence explains the surprising phenomenon of "retour aromatique (aromatic aftertaste)", described by Peynaud (*9*) and well-known to winemakers. The characteristic aromas are initially barely perceptible when you eat a Sauvignon blanc grape or taste the must whereas, a few seconds or even a minute later, there is a much stronger aroma on the rear palate. The expression "aftertaste" is typically used to describe this delayed perception of the aroma of Sauvignon blanc must.

The structure of the precursor in an extract of Sauvignon blanc must was identified thanks to the specific release of volatile thiols by an enzyme capable of cleaving the carbon-sulfur bond (*10*). Its structure was later confirmed by GC/MS analysis in the form of trimethylsylilated derivatives (*11*). In this work, we demonstrated the existence of a new chemical family of sulfur-containing aroma precursors in certain fruit. We have also recently demonstrated that furfural, an aldehyde released by toasted barrel wood, is a precursor of the furanmethanethiol found in certain wines (*12*). This is also the case in coffee (*13,14*). However, its development in wine depends on the fermentation activity of yeast rather than a transformation due to heat. The formation mechanism of this volatile thiol is discussed in relation to the yeast's sulfur metabolism.

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Materials and Methods

Extraction, purification, and assay of the volatile thiols.

The volatile thiols were specifically extracted and assayed using the method described by Tominaga *et al.* (*6,7*).

Assay of hydrogen sulfide in fermenting medium:

The hydrogen sulfide assay used in this study is based on the assay for this compound in "headspace" using GC/FPD. A volume of 50 µL *p*-hydoxypercuribenzoate sulfonium salt (*p*-HMBS) (1 mM in 0.2 M Tris) was added to 500 µL fermenting must in an Eppendorf tube (2mL), then immediately centrifuged (4000 xg, 1min). The supernatant (275 µL) was recovered in a 2mL vial, which was closed with a capsule. A 20 mM glutathion solution (125 µL) was introduced into the vial via a syringe to release H_2S. The vial was then incubated at 30 °C for 30 min. An 125 μL sample was taken from the head-space using a syringe and injected into the GC/FPD, under the conditions described by Lavigne *et al.* (*16*). Calibration charts were prepared (0-5nmol H_2S /vial) using a *p*-HMBS-H_2S complex solution as follows: H_2S gas was put into 1 mL *p*-HMBS (1 mM) in 0.2 M Tris for 5min, then flushed with nitrogen to remove any excess H_2S that had not combined with the *p*-HMBS. In the concentration range (0 to 5 nmol/vial), the standard curve was linear: $[H_2S]$nmol/250μL = 0.0058x – 0.1119, $r^2 = 0.9901$.

Assay of S-3-(hexan-1-ol)-L-cysteine in a fermentation medium

S-3-(hexan-1-ol)-L-cysteine was assayed in a model medium during fermentation using the method described by Tominaga *et al.* (*11*).

Microvinification

The model fermentation medium consist of the following solution: 100 g glucose, 100g fructose, 3 g tartaric acid, 0.3 g citric acid, 0.3 g malic acid, 2 g potassium phosphate, 0.3 g mesoinositol and 64 mg ammonium sulfate, 127 mg asparagine all in 1L of distilled water adjusted to pH 3.3 with solid potassium hydroxide, and vitamin solution (final concentration *d*-biotin 40 μg/L, thiamine hydrochloride 1mg/L, pyridoxine hydrochloride 1mg/L, nicotinic acid 1 mg/L, *d*-panthothenic acid hemicalcium salt 1 mg/L, *p*-aminobenzoic acid 1 mg/L) was added. The fermentation medium for the first microvinification experiment, containing 64mg/L assimilable nitrogen with 15 mg/L added furfural, was supplemented with increasing quantities of nitrogen, by adding either asparagine (90, 140, 190 mg/L) or ammonium sulfate (60, 90, 140 mg/L). The model medium was fermented with *Saccharomyces cerevisiae* (strain Zymaflore VL3).

The fermentation medium for the second microvinification experiment was supplemented with 15 mg/L added furfural, 5 mg/L cysteine, 2g/hL SO2 and increasing quantities of nitrogen, by adding ammonium sulfate (21, 32 and 64 mg/L) and asparagine (42, 63 and 127 mg/L). The model medium was fermented with *Saccharomyces cerevisiae* (strain Zymaflore VL1). For the third microvinification experiment, the fermentation medium with 15 mg/L added furfural was fermented with two yeast strains (*Saccharomyces cerevisiae* VL3c and VS17). The assimilable nitrogen content was ajusted at 191 mgN/L by adding 64 mg/L of ammonium sulfate and 127 mg/L of asparagine.

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Results and Discussion

Developing a specific method for extracting volatile thiols using *p*-hydroxymercuribenzoate (*p*-HMB)

p-HMB has the property of forming reversible combinations with compounds containing a thiol function (*4*) (Figure 1). Volatile thiols can be extracted from an organic extract using a *p*-HMB solution, with which they combine. The original feature of the proposed method (*6,7*) consists of eliminating impurities by purifying the *p*-HMB extract using percolation through an anion exchange column (Dowex 1X2-100). The *p*-HMB, either free or combined with thiols, is fixed on the resin. The column is then rinsed and the volatile thiols are decombined by elution with a cysteine solution.

Extraction of the volatile thiols using the method described above was compared to *p*-HMB extraction only, without percolation through a Dowex column. The peaks for the volatile thiols detected by GC/FPD (figs. 2-a,b) were practically identical, irrespective of the method used. This confirmed that there was no loss of volatile thiols during percolation and rinsing. Contaminant substances were assessed by GC/ FID. Comparison of the two chromatograms (figure 3-a and 3-b) showed that the method using Dowex resin eliminated contaminants (figure 3-a) that could otherwise perturb mass spectrometry identification and assay of the volatile thiols.

Identification of the volatile thiols in Sauvignon blanc wines

The volatile thiols were specifically extracted from 0.5 L Sauvignon blanc wine, using the method described above, and analyzed by GC/O, GC/FPD, and GC/MS under the chromatography conditions described by Tominaga *et al.* (*7*). The two odoriferous zones with box tree and blackcurrant bud aromas, and a zone reminiscent of the aroma of roasted coffee detected in Sauvignon blanc wine by GC/O were easily perceptible on the gas chromatogram (*2,7,17,18*). GC/O had already detected these odoriferous zones reminiscent of box tree in Sauvignon blanc wines, with scores of linear relative retention indices,1459 and 1650 respectively, using a BP-20 (*17*). One of these two compounds had been identified as 4-mercapto-4-methylpentan-2-one (4MMP) (*3*). This highly-odoriferous mercaptoketone has also been reported in Scheurebe wines (*19*) and, more recently, in grapefruit juice (*20*).

The compound responsible for the second odoriferous zone reminiscent of box tree, but with more complex nuances of tropical fruit, was identified as 3-

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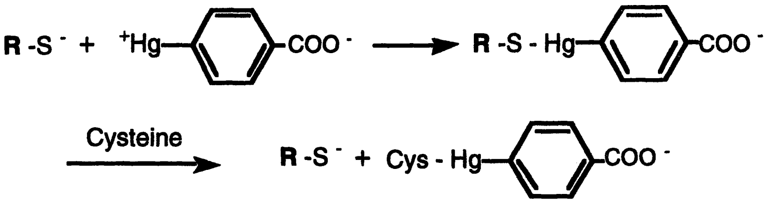

Figure 1. Reversed combination of volatile thiols with p-HMB.

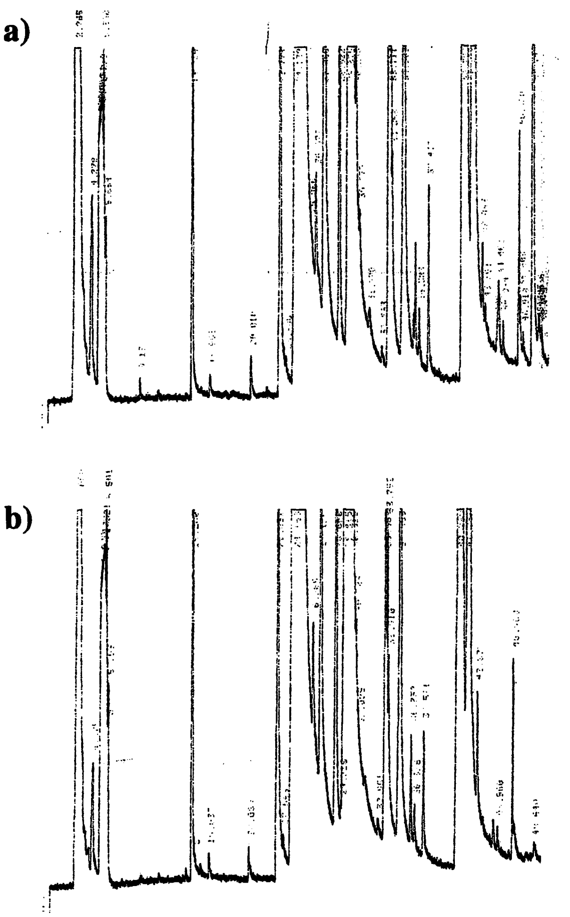

Figure 2. Comparison of the GC/FPD analyses of the volatile thiols in a Sauvignon blanc wine extracted using two different methods: a) without percolation, b) with percolation.

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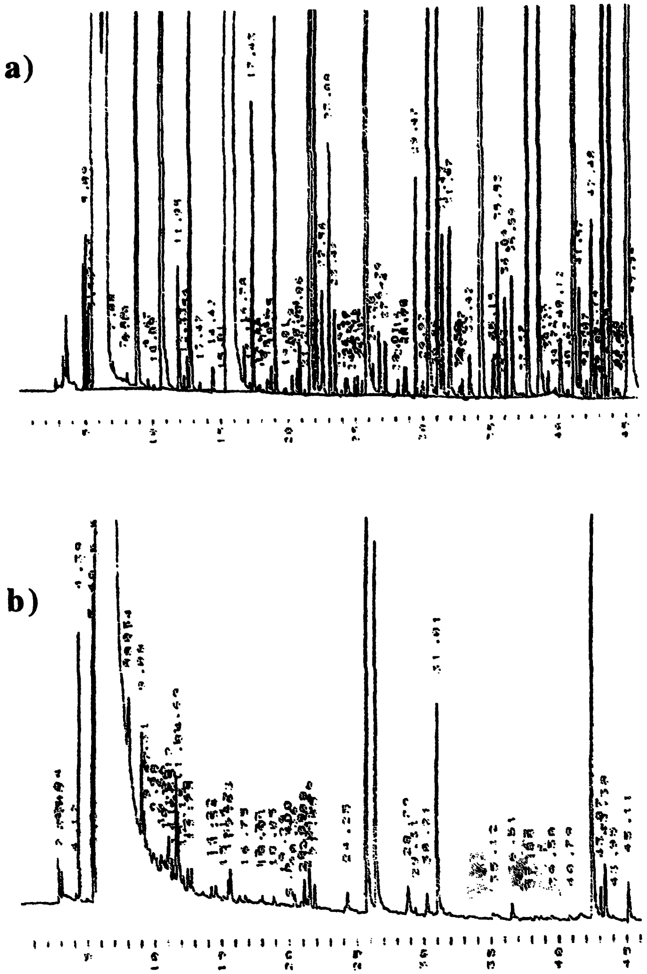

Figure 3. Purity assessment by GC/FID of the volatile thiol extract obtained by two different methods: a) without percolation, b) with percolation.

mercaptohexyl acetate in 1996 (*21*). The new specific extraction method for volatile thiols made it possible to identify three new mercaptoalcohols in Sauvignon blanc wines: 4-mercapto-4-methylpentan-2-ol (4MMPOH), 3-mercapto-3-methylbutan-1-ol (3MMB), and 3-mercaptohexan-1-ol (3MH) (*22*) (Table 1). None of these 4 compounds had previously been found in wine. One of these volatile compounds, 3-methyl-3-mercaptobutan-1-ol, is also found in roasted coffee (*23*). Engel and Tressl identified 3-mercaptohexan-1-ol and its acetate in passion fruit (*24*). To our knowledge, 4-mercapto-4-methylpentan-2-ol had never previously been identified in nature.

The odoriferous compound corresponding to roasted coffee in certain red and white wines was more recently identified as 2-furanemethanethiol (2FM), using the more specific method for extracting volatile thiols (*7*). This volatile thiol is well-known in the aroma field for its distinctive roasted coffee odor and extremely low perception threshold. It had previously been identified in several food products: coffee, meat stock, and canned tuna (*25-28*). We report here that this volatile thiol has been identified for the first time in certain *Vitis vinifera* wines.

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Assay of the volatile thiols identified in wines made from several white and red *Vitis vinifera* grape varieties

The 6 volatile thiols in several Sauvignon blanc wines from Sancerre and Bordeaux were assayed in SIM mode (*6,7*) (Table 2). The 4MMP and 3MH concentrations were higher than their respective perception thresholds in all wines analyzed. Consequently, the aromatic indices (concentrations found in wine/perception threshold in model solution) (*29*) for these compounds was considerably higher than 1. 3MHA was no longer present in wines from older vintages. 4MMPOH and 3MMB concentrations were always below the perception threshold, irrespective of the age of the wines analyzed.

It is, therefore, undeniable that 4MMP, with its box tree odor, and 3MH, reminiscent of grapefruit and passion fruit, have an impact on the aroma of Sauvignon blanc wines. 3MHA contributes to the box tree aroma in some young wines, but it hydrolyzes rapidly during bottle aging, or even barrel-aging on the lees (*15*). It is, however, unlikely that 4MMPOH and 3MMB contribute to the aroma of wines made from this grape variety.

4MMP is also found in large quantities in the two plants, box tree and broom (*30*). Thus, the terms "box tree" and "broom", long used to describe these wines, do, in fact, correspond to a chemical reality.

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Table I. Structure of the volatile thiols identified in Sauvignon blanc wines.

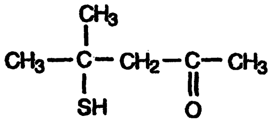
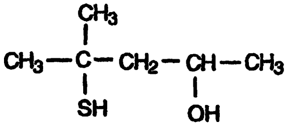
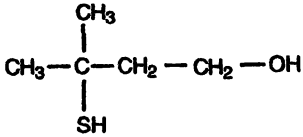
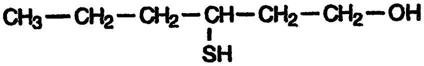
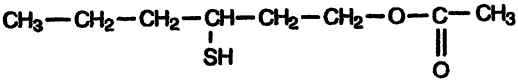
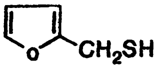
Compound	*Structure*
4-Mercapto-4-methylpentan-2-one (4MMP)	 $CH_3-C(CH_3)(SH)-CH_2-C(=O)-CH_3$
4-Mercapto-4-methylpentan-2-ol (4MMPOH)	 $CH_3-C(CH_3)(SH)-CH_2-CH(OH)-CH_3$
3-Mercapto-3-methylbutan-1-ol (3MMB)	 $CH_3-C(CH_3)(SH)-CH_2-CH_2-OH$
3-Mercaptohexan-1-ol (3MH)	 $CH_3-CH_2-CH_2-CH(SH)-CH_2-CH_2-OH$
3-Mercaptohexyl acetate (3MHA)	 $CH_3-CH_2-CH_2-CH(SH)-CH_2-CH_2-O-C(=O)-CH_3$
2-Furanmethanethiol (2FM)	

Table II. Organoleptic incidence of the volatile thiols identified in Sauvignon blanc wines.

Compound	*Olfactory description*	*Perception threshold (ng/L)*	*Concentration in wines (ng/L)*	*Aromatic index*[2]
4MMP[1]	Box tree, Broom	0.8	0-40	5-55
3MH	Grapefruit Passion fruit	60	200-5000	10-200
3MHA	Box tree Passion fruit	4.2	0-500	0-200
4MMPOH	Citrus zest	55	0-150	0-2
3MMB	Cooked leeks	1500	30-150	0
2FM	Roasted coffee	0.4	0-20	0-50

[1]Darriet et al., 1995; [2]Aromatic index = concentration in wine/perception threshold.

These volatile thiols are present in wines made from other *Vitis vinifera* grape varieties (*31*). For example, 4MMP and 3MH are present in particularly high concentrations in Alsatian Muscat d'Alsace and Gewurztraminer wines, respectively (Figure 4).

Concentrations of 3MH are also considerably higher than the perception threshold in Petit Manseng, Gros Manseng, Colombard, and botrytized Semillon wines (*31*) (Table 3). The presence of 3MH has also been reported in red Cabernet and Merlot wines (*32,33*). Apparently, however, this volatile thiol has very little impact on the aroma of red wines. On the other hand, 3MH has recently been shown to add a fruity nuance to some Cabernet and Merlot rosé wines (results not shown) (*34*). 3MHA has been identified in young white Petit Manseng, Gros Manseng, and Colombard wines.

White wines fermented in new barrels have a high 2FM content, irrespective of the grape variety, as shown for the Sauvignon blanc wines in figure 6. This volatile thiol is only present in trace amounts in white wines fermented in stainless-steel vats. The red wines analyzed did not systematically contain 2FM. Higher concentrations were found in wines aged in new barrels (Figure 5) (*12*). The concentration of this volatile thiol in red wines is, however, always lower than in white wines (Figure 6). However the concentration/perception threshold ratio of 2FM in certain red wines is quite high, making it likely that this compound contributes "roasted coffee" nuances to their aroma. Marchand *et al.* (*35*) recently reported much higher concentrations of this volatile thiol in certain red Pomerol and St. Emilion wines (70-350 ng/L).

Enzyme release of certain volatile thiols by β-lyase from *Eubacterium limosum*

The surprising "aromatic aftertaste" phenomenon mentioned in the introduction is due to an aroma precursor in Sauvignon blanc. Previous research (*8,18*) had shown quite clearly that 4MMP could not be released from its precursor by the glycosidases capable of hydrolyzing glycosides, the precursors of monoterpenols. This indicates that the precursor of 4MMP is probably not a glycoside.

Among the enzyme activities capable of cleaving a carbon-sulfur bond and releasing a thiol, our attention was attracted by a S-cysteine conjugate ß-lyase (EC4.4.1.13). This enzyme, produced by an intestinal bacterium, *Eubacterium limosum* (*36,37*), catalyzes the cleaving of the thioether bond in a number of conjugates (S-alkyl- and S-aryl-) of L-cysteine, releasing mercaptan, as well as ammonium and pyruvic acid (Figure 6). The β-lyase activity of *Eubacterium limosum* is already used in aroma production to synthesize *p*-mentha-8-thiol-3-one from pulegon (*38*).

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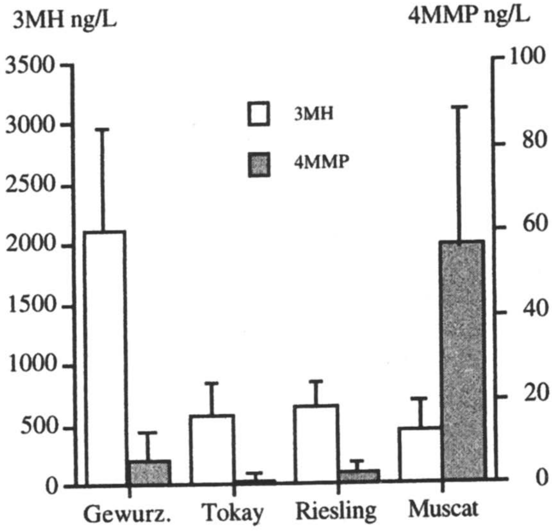

Figure 4. 3MH and 4MMP concentrations in wines made from Alsace grape varieties (n=5).

Table III. Volatile thiol concentrations (ng/L) in wines made from several white grape varieties (n=4)

Varieties	*4MMP*	*A3MH*	*3MH*	*2FM*
Petit manseng	0	5-100	1000-5000	10-60
Gros manseng	0	50-600	5000-10000	0
Colombard	0	20-60	400-1000	0
Botrytized Semillon	8-40	0	4000-5000	0-20
Cabernet, Merlot (Red Bordeaux)	0	<5	200-500	2-25

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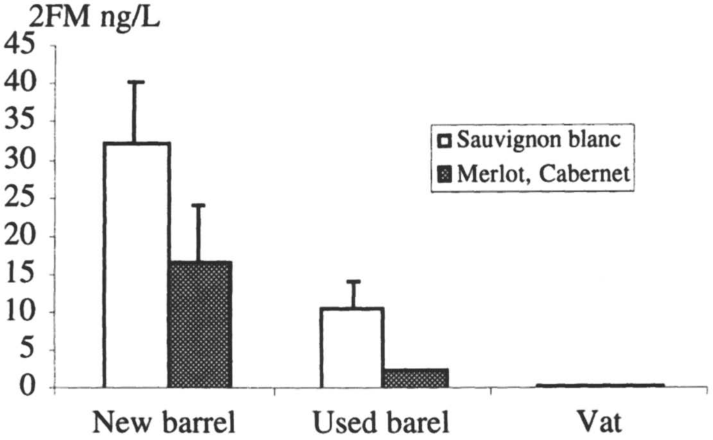
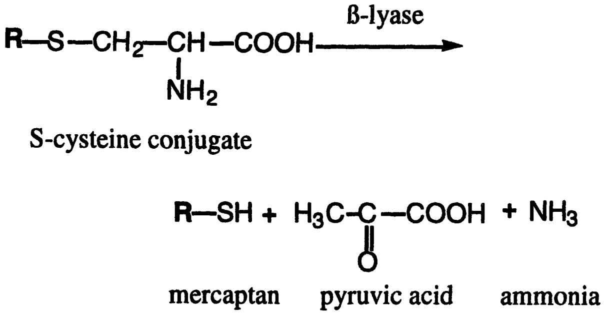


Figure 5. Contribution of oak barrels to 2FM formation in Sauvignon blanc and red Bordeaux wines (n=4).



$$R{-}S{-}CH_2{-}\underset{\displaystyle NH_2}{\underset{|}{CH}}{-}COOH \xrightarrow{\beta\text{-lyase}} R{-}SH + H_3C{-}\underset{\displaystyle O}{\underset{\|}{C}}{-}COOH + NH_3$$

Figure 6. Enzymatic release of volatile thiol from S-cysteine conjugate.

A crude extract containing sulfur flavor aroma precursors (CESFPs), prepared from Sauvignon blanc juice by percolation on a C_{18} grafted silica column and eluted with ethanol (*8*), was subjected to the action of a cell-free extract from ***Eubacterium limosum*** (*10*). After 15 minutes incubation at 30°C, the reagent medium gave off a strong odor reminiscent of Sauvignon blanc aromas. The volatile compounds formed were analyzed (Figure 7). Peaks 1, 2, and 3 were identified using mass spectrometry. 4MMP (peak 1), 4MMPOH (peak 2), and 3MH (peak 3) were formed by the action of the cell-free extract on the CESFPs. If the bacterial extract has been deactivated by heat treatment, no thiols are released (*10,11*).

In view of the specific activity of the β-lyase present in the bacterial preparation, it was likely that the precursors of these various thiols in fruit were S-cysteine conjugates.

Identifying a new type of sulfur flavor precursor in certain fruit: S-cysteine conjugate

The S-cysteine conjugates were directly identified as follows. The CESFPs (corresponding to an initial quantity of 30 L must) including the sulfur flavor precursors to be identified were purified by percolation through a Chelating Sepharose 4B column, which has the property of fixing certain amino acids via the intermediary of chelated copper (*39*). The retained fraction was eluted with HCl (50 mM). When the eluate had been evaporated dry, the residue was extracted with ethanol and dried. The purified aroma precursors, in the form of trimethylsilylated derivatives, were analyzed by GC/MS. This analysis led to the identification of the structures of the precursors of three volatile thiols: *S*-4-(4-methylpentan-2-one)-L-cysteine, *S*-4-(4-methylpentan-2-ol)-L-cysteine, *S*-3-(hexan-1-ol)-L-cysteine (Figure 8) (*11*). *S*-3-(hexan-1-ol)-L-cysteine was recently identified in Cabernet and Merlot must in the same way (40).

The amplification of the varietal aroma of this grape variety by the yeast's (*Saccharomyces cerevisiae*) fermentation mechanism results from the breakdown of cysteinylated aroma precursors from the grapes, leading to the formation of the corresponding volatile thiols, as shown in figure 9.

The fact that a cysteinylated precursor of 3MH had been found in Sauvignon blanc grapes led us to check whether such a compound was also present in passion fruit, as this fruit contains large quantities of 3MH (*24*). Passion fruit juice extract was therefore subjected to the same chromatography analyses. An S-cysteine conjugate, *S*-3-(hexan-1-ol)-L-cysteine, was also found in this fruit (*41*). This confirms that S-cysteine conjugates constitute a new family of aroma precursors in certain fruit.

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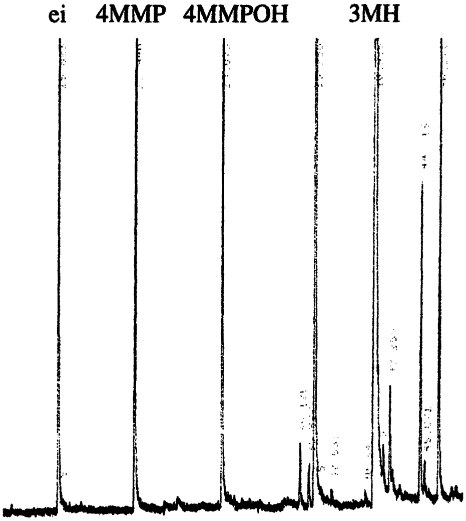

Figure 7. Release of 4MMP, 4MMPOH and 3MH from Sauvignon blanc must extract by enzyme action of cell-free extract from Eubacterium limosum.

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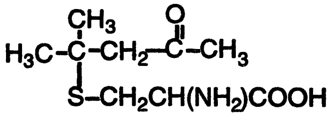
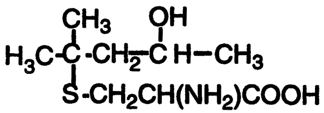


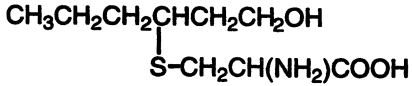
$H_3C-C(CH_3)(S-CH_2CH(NH_2)COOH)-CH_2-C(=O)-CH_3$

S-4-(4-methylpentan-2-one)-L-cysteine



$H_3C-C(CH_3)(S-CH_2CH(NH_2)COOH)-CH_2-CH(OH)-CH_3$

S-4-(4-methylpentan-2-ol)-L-cysteine



$CH_3CH_2CH_2CH(S-CH_2CH(NH_2)COOH)CH_2CH_2OH$

S-3-(hexan-1-ol)-L-cysteine

Figure 8. Structure of the three Sauvignon blanc aroma precursors: S-cysteine conjugate.

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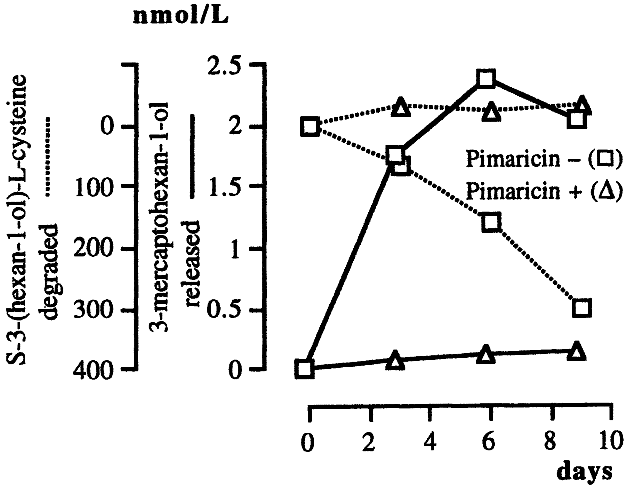

Figure 9. Release of 3-mercaptohexan-l-ol during alcoholic fermentation from a model medium to which synthesized S-3-(hexan-1-ol)-L-cysteine was added in the absence ( ) and presence ( ) of pimaricin.

Both 3-mercaptohexanol and its cysteinylated precursor are present in *Passiflora edulis* (passion fruit). However in *Vitis vinifera* L. cv. Sauvignon blanc grapes, the volatile thiols characteristic of varietal aroma are mainly present in conjugate form. They are released during alcoholic fermentation following the breakdown of the corresponding S-cysteine conjugates by yeast. This previously unexplained amplification of grape aroma during fermentation has now received a molecular explanation. In passion fruit, the conjugate is probably converted into free thiol either by acid hydrolysis, or by an endogeneous enzyme, such as a β-lyase.

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Formation Mechanism of 2FM in White Wines

In this report, we showed quite clearly that the 2FM identified in white wines was associated with barrel-fermentation. The formation mechanism of this thiol has always been associated with heat-induced transformations such as roasting coffee or cooking food (*42,43*). However, the quantities of 2FM formed in the barrel staves when they are toasted are too small to explain the 2FM content in barrel-fermented wines (*7*). Furthermore, the fermentation process never reaches the high temperatures required.

An assay of the furfural and 2FM in wines made from Sauvignon blanc must fermented in barrels of different origins and ages showed a significant correlation between these two compounds (Figure 10) (*12*). 2FM is apparently formed in white wines from the furfural released by the barrels during alcoholic fermentation. In fact, 2FM is only formed in fermented media in the presence of furfural. Media containing furfural and pimaricin, or no furfural at all, do not contain 2FM (results not shown). The formation of 2FM in white wines, therefore, depends on the presence of furfural combined with the fermentation activity of yeast.

The available nitrogen content of the must has a direct impact on the amount of H_2S produced by the yeast (*44-47*). It is difficult to ferment nitrogen-deficient must and excessive amounts of H_2S are sometimes produced. This means that the available nitrogen content of the must also has an effect on 2FM production. A model fermentation medium with added furfural (15 mg/L) was fermented using VL3c and the available nitrogen content was increased by adding asparagin or ammonium sulfate.

Must is considered to have a high available nitrogen content at values above 200 mg/L. Enhancing the nitrogen content of a model medium by adding asparagin clearly led to a decrease in 2FM production (Figure 11). However, increasing the nitrogen content by adding ammonium sulfate had less effect on 2FM production (Figure 11). In this case, the 2FM content remained stable, irrespective of the quantity of nitrogen added.

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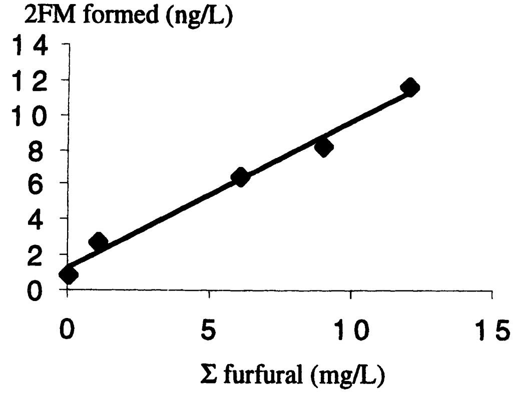

Figure 10. Relationship between the furfural released by the barrels and the FFT content of the white wines after fermentation.

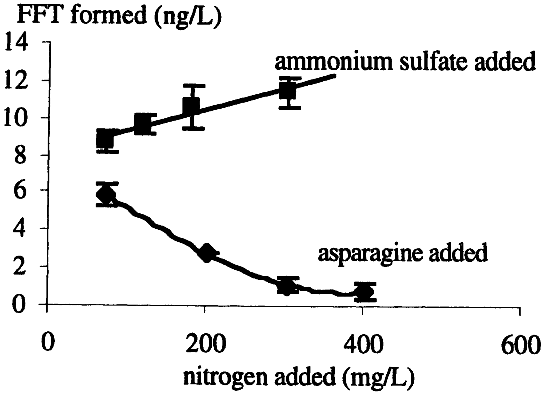

Figure 11. Changes in FFT content according to the total nitrogen content of a fermentation medium, adjusted by adding asparagines and ammonium sulfate (n=2).

In a nitrogen-deficient must, amino-acid synthesis is slowed down and the sulfides produced by the yeast are released into the medium as H_2S. Production of the HS^- anion in excess of the amounts required for yeast's protein synthesis promotes the formation of 2FM, as shown in figure 11. In practice, ammonium sulfate is often used to correct excessive H_2S production. As these adds both sulfur and nitrogen to the must (*48*), it is logical that 2FM production should be less impacted, even if the nitrogen level is high.

We were also interested to know whether the reaction substituting a SH group for the furfural's carbonyl group took place inside or outside the yeast cells.

A fermentation medium with 15 mg/L added furfural was supplemented with increasing quantities of assimilable nitrogen by adding ammonium sulfate (21, 32, 64 mg/L) and asparagine (42, 63 and 127 mg/L). During alcoholic fermentation, H_2S liberation was stronger in a medium containing low quantity of nitrogen (Figure 12). Average H_2S concentration was much higher when nitrogen content was lower (Figure 13). However, the 2FM content remained stable, independently of the quantity of H_2S liberated in the fermentation medium.

An another example was shown in figs. 14 and 15. Synthetic medium (191 mg/L assimilable nitrogen) with 15 mg/L added furfural was fermented with two yeast strains (VL3c and VS 17). Although both strains had the same aptitude for releasing H_2S during alcoholic fermentation (Figure 14), the 2FM content at the end of fermentation in the synthetic medium fermented with VL3c was twice higher than in the sample with VS17 ($P<0.001$) (Figure 15). It seems likely no-relation between the 2FM formation and H_2S content in a fermentation medium. Those results suggested that 2FM is formed inside the yeast cell, then released.

Conclusion

Sauvignon blanc is currently the only non-Muscat grape variety whose aroma can be partially interpreted by chemical analysis. Several sulfur compounds, responsible for certain characteristic nuances of the aroma of wines made from this grape variety, have been identified, as well as their odorless precursors in grapes. However, these few key molecules are certainly not the only ones involved in the complex aromas of the finest wines. Of course, wines with a marked varietal aroma always have higher 4MMP and 3MH contents, but other compounds are certainly involved. These may also be volatile thiols.

We are currently studying the mechanism by which the yeast transforms the cysteinylated precursor into aroma. Work is also in progress to elucidate the formation of 2FM in red wines during barrel-aging.

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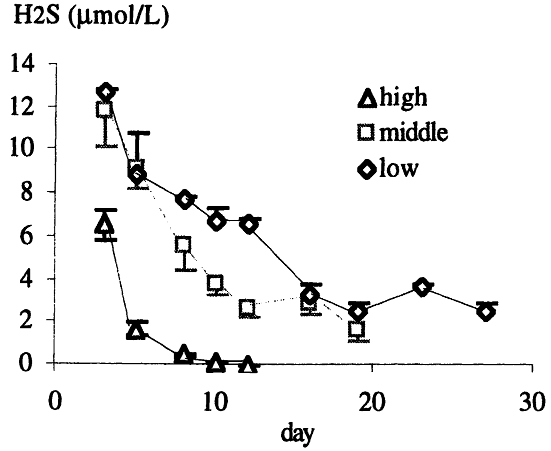

Figure 12. Evolution of H_2S concentration during alcoholic fermentation according to the total nitrogen content of a fermentation medium, adjusted by adding ammonium sulfate (n=3).

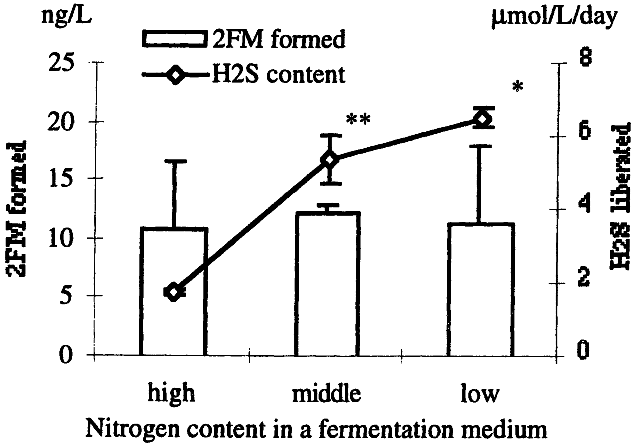

Figure 13. Influence of H_2S content in a fermentation medium on the 2FM formation (n=3). $^{**}P<0.01$, $^{*}P<0.05$.

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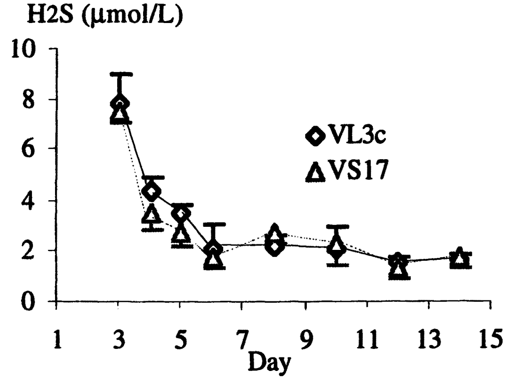

Figure 14. Development of H_2S during alcoholic fermentation in a model medium fermented with two yeast strains (n=3).

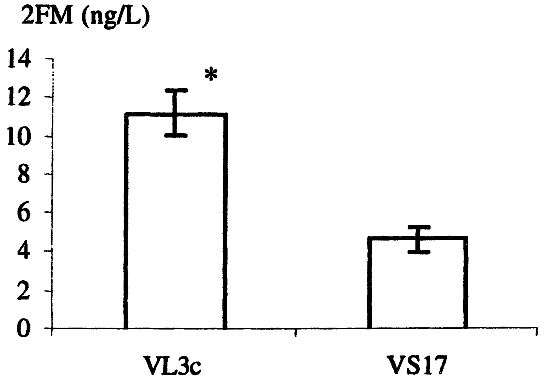

*Figure 15. Comparison of the 2FM content at the end of alcoholic fermentation in a synthetic medium fermented with two yeast strains (n=3). *P<0.001.*

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Chapter 25

Activity-Guided Screening and Identification of Natural "Cooling" Compounds Formed from Carbohydrates and L-proline in Beer Malt

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Gel permeation chromatography (GPC) of the solvent-extractables isolated from a thermally treated glucose/L-proline mixture and sensory analysis of the fractions collected led to the discovery of the presence of "cooling" compounds in Maillard reactions. To characterize the compounds imparting this oral cooling sensation, the Taste Dilution Analysis was applied to the cooling-active GPC fraction by determining the taste threshold of reaction products in serial dilutions of HPLC fractions. MS, NMR, [^{13}C] labeling experiments, followed by synthesis led to unequivocal identification of 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC), 5-methyl-2(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC) and 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (3(2*H*)-DMPF) as the most intense "cooling" compounds formed from hexoses. Comparative studies on pentose/L-proline mixtures led to the identification of the odorless 5-methyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (3(2*H*)-MPF), exhibiting a "cooling" sensation at low concentrations of 1.5-3.0 mg/kg (water), as one of the most active "cooling" agents reported so far. To the best of our knowledge, these are the first Maillard reaction products reported to cause intense cooling sensations by degustation. Finally, the detection of 5-MPC, 3-MPC, 3(2*H*)-DMPF, and 3(2*H*)-MPF in dark roasted beer malts verified their natural occurence in thermally processed foods and demonstrated 3(2*H*)-MPF as the most active, odorless cooling agent reported so far in nature.

Malt is produced by steeping and germination of cereals, in particular barley, followed by a kiln-drying and/or a roasting process. Depending on the processing parameters during malt manufacturing, a variety of malt with different aroma, taste and color attributes are obtained. Thermal reactions, in the particular Maillard reaction between reducing carbohydrates and amino acids liberated from corresponding biopolymers during germination, are chiefly responsible for the development of the unique aroma and taste as well as the typical brown color of

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

malt. Although not used as a food as such, malt is used for centuries in the manufacturing of whiskey and beer, and gain growing interest as flavoring and coloring material in, e.g. breadmaking or the manufacturing of breakfast cereals.

Although the consumer acceptance of these foods is strongly influenced by a balanced interplay between the aroma-active (perceived in the nose) as well as the taste-active components (detected on the tongue), the information available on sapid taste compounds present in dark malt is as yet very limited. Diketopiperazines, such as, e.g. cyclo(Phe-Pro), cyclo(Leu-Pro), cyclo(Pro-Pro), cyclo(Val-Pro) and cyclo(Ile-Pro), which are formed by dimerization of amino acids or peptides, have been reported as bitter tastants in roasted malt (*1*). To answer the question whether besides such diketopiperazines taste compounds are also formed from carbohydrates and amino acids during malt manufacturing, numerous studies have been focused on the identification of sensorially active reaction products formed in food-related, Maillard-type model mixtures. Because steeping and germination of barley produce L-proline and glucose as the quantitatively predominating Maillard precursors prior to the kilning and roasting process, the reaction between these components has been extensively investigated in the last decades to model the flavor formation during the manufacturing of roasted malts. Because most of these identification experiments have been primarily focused on the quantitatively predominating products formed, rather than selecting the target compounds with regard to taste-activity, only a few taste compounds have been identified so far, e.g. pyrrolidino-hexose-reductones (*2-4*), pyrrolidines (*5*) and cyclopent[*b*]azepin-8-ones (*5-7*).

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In order to bridge the gap between pure structural chemistry and human taste perception, a more straightforward technique, the so-called Taste Dilution Analysis (TDA) has recently been developed, which is based on the determination of the detection threshold of taste compounds in serial dilutions of HPLC fractions (*8*). This novel bioassay offers the possibility to rank food components according to their relative taste impact and has proved to be a powerful technique for the identification of key taste compounds; e.g. the novel 3-(2-furyl)-8-[(2-furyl)methyl]-4-hydroxymethyl-1-oxo-1*H*,4*H*-quinolizinium-7-olate, exhibiting an extraordinarily low detection threshold of 0.00025 mmol/kg water, was successfully identified as the most intense bitter tasting compound formed during thermal treatment of pentoses and primary amino acids (*8*).

Until now, all the taste-active compounds detected in Maillard reaction mixtures or in roasted malts showed bitter taste qualities only. However, no information is available on the chemical structures of more desirable tastants. The aim of the present investigation was, therefore, to identify taste compounds exhibiting desirable sensory qualities in heated carbohydrate/L-proline mixtures and to verify their natural occurrence in processed cereals, i.e. in dark malt.

Experimental

Materials

The following compounds were synthesized as following the procedures reported recently: 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone (*9*), 3-deoxy-2-hexosulose (*10*), 3,5-dihydroxy-2-methyl-5,6-dihydropyran-4-one (*11*), 3-MPC (*12*), 5-MPC (*12*), 3(2*H*)-DMPF (*12*), and 3(2*H*)-MPF (*13*). Malts were supplied by the German brewing industry.

Thermally treated carbohydrate/L-proline mixture

Glucose (200 mmol) or xylose (200 mmol), respectively, was dry-heated in the presence of L-proline (200 mmol) for 20 min at 190°C, cooled to room temperature, suspended in hot water (1.5 L) and filtered. The solvent extractable reaction products were isolated with CH_2Cl_2 and separated by gel permeation chromatography.

Gel permeation chromatography (GPC)

The solvent extractables of the glucose/L-proline mixture (2.89 g) were separated by gel permeation chromatography on Sephadex LH-20 (750 mm × 55 mm, Pharmacia, Upsala, Sweden) using a mixture (75/25, v/v; 3 mL/min) of methanol and aqueous ammonium formate (50 mmol/L; pH 3.5) as the eluent (*12*). Monitoring the effluent at 300 nm, ten fractions (Figure 1) were collected, freed from solvent at 30°C *in vacuo* (45 mbar), and then freeze-dried. The material of each fraction was dissolved in tap water and used for sensory analysis (Table I).

Thermally treated mixtures of hexose-derived intermediates and L-proline

Mixtures of L-proline (10 mmol), the hexose intermediates (10 mmol) given in Table II, and Al_2O_3 (10 g, neutral) were dry-heated for 10 min at 180°C. After cooling, the mixtures were taken up in hot water (500 mL), and the solvent extractables were analyzed by HPLC/taste dilution analyses (*12*).

HPLC/taste dilution analysis (HPLC/TDA)

An aliquot (200 mg) of fraction V (Figure 1), the solvent-extractable fraction of the cyclotene/L-proline mixture or the 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone/ L-proline mixture (data not given), respectively, was dissolved in methanol, and analyzed by RP-HPLC. The effluent was separated into 30 (GPC fraction V; Figure 2) or 16 fractions (cyclotene/L-proline mixture; Figure 4), respectively, on which the HPLC/taste dilution analysis was applied as recently reported (*12*).

[^{12}C/^{13}C] Labeling experiment

Glucose (0.5 mmol), [$^{13}C_6$] glucose (0.5 mmol) and L-proline (1.0 mmol) were mixed and dry-heated for 15 min at 190°C. After solvent extraction and column chromatography, HRGC/MS revealed the isotopomeric patterns of the molecular ions of the "cooling" compounds detected by HPLC/TDA of fraction V (*12*).

Identification of "cooling" compounds in dark malts

Ground malt (50 g) was stirred overnight with dichloromethane (2 × 400 mL), the combined organic layers were concentrated to about 50 mL, and the volatile components were isolated by high vacuum distillation at 35°C (*14*). After pre-separation by column chromatography on Al_2O_3, 3-MPC, 5-MPC, 3(2*H*)-DMPF, and 3(2*H*)-MPF were identified and quantified by comparison of the

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retention times, the mass spectra as well as the peak areas with those obtained for the synthetic reference compounds (*12*).

Results and Discussion

HPLC analysis of the solvent-extractable fraction isolated from a roasted equimolar mixture of glucose and L-proline showed that a tremendous multiplicity of reaction products had been formed. To sort out the strongly taste-active compounds from the bulk of less taste-active or tasteless substances, the reaction products were separated from the high-molecular, melanoidin-type material by means of gel permeation chromatography on Sephadex LH-20. Monitoring the effluent at 300 nm, the GPC chromatogram, displayed in Figure 1, was recorded and ten fractions (Fractions I to X) were collected separately.

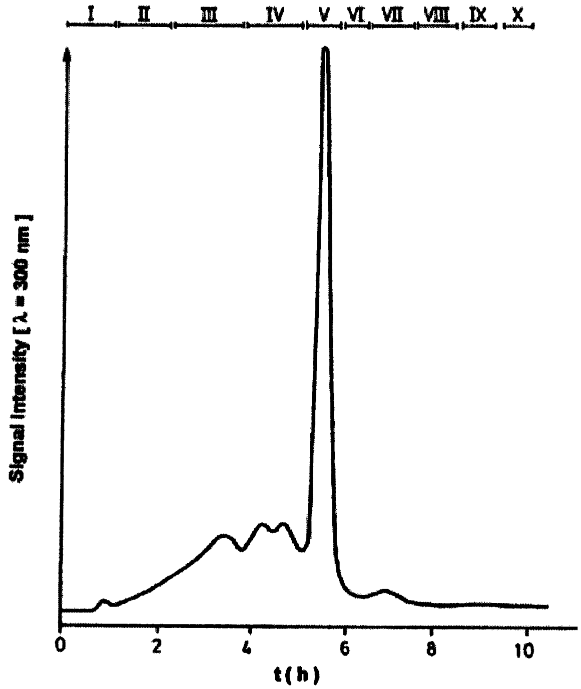

Figure 1. GPC chromatogram of the solvent-extractable fraction of a dry-heated glucose/L-proline mixture

In order to evaluate their taste activity, these GPC fractions were freeze-dried, the residues were taken up in tap water and then presented to a trained sensory panel which was asked to judge the taste qualities of these fractions by gustation in a triangle test (*12*). Whereas fraction **I** and fractions **VIII-X** did not show any taste impact, fractions **II** to **VI** tasted bitter (Table I). It was, however, interesting to notice that fraction **V**, showing the most intense absorption at 300 nm (Figure 1), in addition to the bitter taste, imparted a significant "cooling" effect to the tongue of the panelists (*12*). Because the presence of "cooling" compounds in Maillard reactions has not previously been reported, the following identification experiments focused on fraction **V**.

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Table I. Taste qualities and yields of fractions obtained by GPC of the solvent-extractable reaction products formed from glucose and L-proline

fraction no.[a]	*yield*[b]		*taste quality*
	[mg]	*[%]*	
I	202	6.99	n.d.
II	428	14.81	bitter
III	720	24.91	bitter
IV	855	29.58	bitter
V	415	14.36	bitter, "*cooling*"
VI	120	4.15	bitter
VII	53	1.83	bitter
VIII	28	0.97	n.d.
IX	10	0.35	n.d.
X	<1	<0.01	n.d.

[a] Number of GPC-fraction refers to Figure 1. [b] The yields relate to the amount of material (2.89 g) applied onto the column. n.d.: no taste detectable.

HPLC/Taste dilution analysis of fraction V

To investigate the reaction products present in fraction V, first, this fraction was chromatographed by RP-HPLC (Figure 2, left).

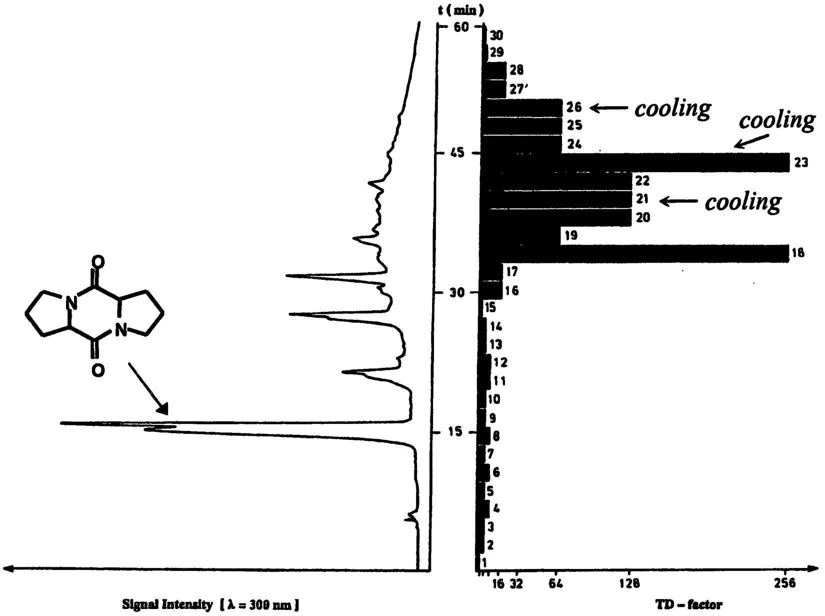

Figure 2. RP-HPLC chromatogram (left) and Taste Dilution (TD)-chromatogram (right) of fraction V obtained by GPC of the glucose/L-proline mixture

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In order to locate the taste-active compounds in fraction V and to rank them in their relative taste impacts, we applied the Taste Dilution Analysis onto fraction V. To achieve this, an aliquot of this fraction was chromatographed by RP-HPLC (Figure 2, left) and the effluent was separated into 30 fractions, which were freeze-dried, then made up with water to 1 mL, and, finally, stepwise 1:1 diluted with water. These serial dilutions were presented in order of increasing concentrations to trained sensory panelists, who were asked to evaluate the taste quality and to determine the threshold dilution at which a taste difference between the diluted fraction and two blanks (tap water) could just be detected (*12*). Since this so-called Taste Dilution (TD) factor, determined for each fraction, is related to its taste activity in water, the HPLC fractions **V-1** to **V-30** were ranked in their relative taste impacts (Figure 2, right).

As fractions **V-18** and **V-23** had TD factors of 256, they exhibited by far the highest taste intensity. Both fractions exhibited a bitter taste, but fraction **V-23**, in addition, imparted an intense cooling sensation to the oral cavity. In addition, significant cooling activity was detectable in fractions **V-21** and **V-26**, whereas most of the other HPLC fractions evaluated with higher TD factors were judged only as bitter tasting with or without astringency (*12*). Fraction **V-8**, which contained the well-known bitter-tasting diketopiperazine of L-proline as the quantitatively predominating reaction product in GPC fraction V, was evaluated with a TD factor of 8 only, thus ruling out this compound as a key contributor of the taste of the roasted Maillard mixture, and demonstrating the taste dilution analysis as a powerful activity-guided tool to identify the most sensory active compounds in complex materials such as foods.

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Structure determination of hexose-derived "cooling" compounds

To characterize the Maillard reaction products causing the cooling sensation, fractions **V-21**, **V-23** and **V-26** were separately collected and analyzed by LC/MS and HRGC/MS. In fraction **V-21**, a reaction product was detected exhibiting cooling activity upon degustation and showing a molecular mass of 181 Da. Analysis of fractions **V-23** and **V-26** revealed two major reaction products, both of which had a molecular weight of 165 Da and caused a pronounced cooling sensation in the oral cavity. To obtain sufficient material for ^{1}H and ^{13}C NMR analysis, the following experiments aimed to produce these "cooling" compounds more directly by reacting potential hexose-derived precursors with L-proline. In order to characterize such carbohydrate intermediates, a labeling experiment was performed with an equimolar mixture of natural [^{13}C]-abundant glucose, and [$^{13}C_6$]-glucose (*12*). After chromatographic isolation, HRGC/MS analysis of the "cooling" compounds in fractions **V-21**, **V-23** and **V-26** revealed a 1+1 mixture of the non-labeled and six-fold labeled isotopomers, i.e., molecular ions with *m/z* 181 and with a shift of six a molecular ion with *m/z* 187 was found for the "cooling" compound in fraction **V-21** (A in Figure 3). GC/MS of the "cooling" compounds in fractions **V-23** (B in Figure 3) and **V-26** (C in Figure 3), respectively, revealed $[M]^+$ ions with *m/z* 165 and 171, corresponding to the natural [^{13}C] abundant molecules and the six-fold [^{13}C]-enriched isotopomers, respectively (*12*).

The lack of isotopomeric mixing clearly indicated that the "cooling"-active compounds are formed without carbon skeleton fragmentation from the original C_6-carbon chain of the hexose. In order to identify potential precursors and to generate the "cooling" compounds in higher yields, L-proline was reacted with various well-known carbohydrate intermediates with C_6-backbone as detailed in Table II (*12*).

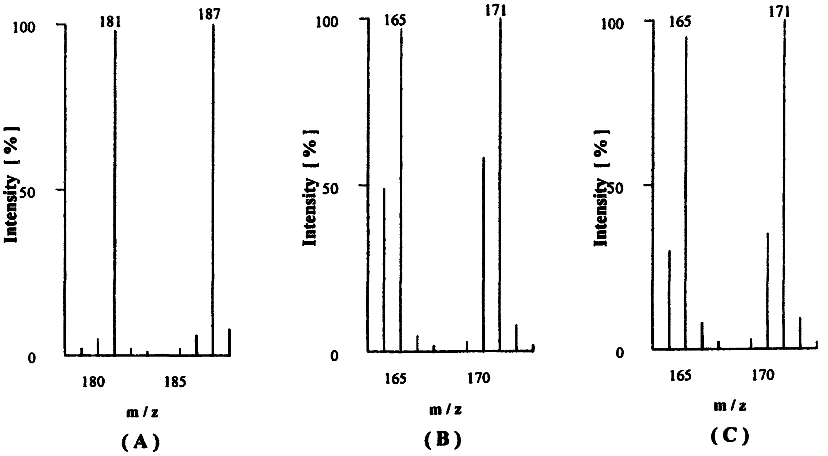

Figure 3. Isotopomeric pattern of the molecular ions (MS-EI) of "cooling" compounds in HPLC fractions V-21 (A), V-23 (B), and V-26 (C) obtained from a thermally treated mixture (1/1/2) of natural abundant [^{13}C] glucose, [$^{13}C_6$] glucose and L-proline

HPLC analysis and degustation of the fractions collected revealed that the "cooling" compounds detected in fractions **V-23** and **V-26** were exclusively formed in the reaction mixture containing the C_6-intermediate 2-hydroxy-3-methyl-2-cyclopenten-1-one (cyclotene), whereas 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone favored the formation of the "cooling" compound detected in fraction **V-21** (*12*). To a minor extent also the 2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone, which is known to generate the 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone, produced the target compound in fraction **V-21**.

Table II. Cooling effects detected in HPLC fractions from heated mixtures of hexose degradation products and L-proline

Hexose degradation product [a]	*cooling effect in fraction*		
	V-21	*V-23*	*V-26*
3-deoxy-2-hexosulose	-	-	-
3,5-dihydroxyl-2-methyl-pyran-4-one	-	-	-
5-(hydroxymethyl)-furan-2-aldehyde	-	-	-
2,4-dihydroxy-2,5-dimethyl-3(2*H*)-furanone	+	-	-
2,5-dimethyl-4-hydroxy-3(2*H*)-furanone	++	-	-
2-hydroxy-3-methyl-2-cyclopenten-1-one	-	++	++

[a] Hexose intermediates (10 mmol), L-proline (10 mmol) and Al_2O_3 (10 g) were mixed and dry-heated for 10 min at 180°C. After cooling, the mixtures were taken up in hot water (500 mL), and the solvent extractables were analyzed by HPLC/degustation.

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Because cyclotene was elucidated as the precursor for the two "cooling" compounds in fractions **V-23** and **V-26**, the following identification experiments were focused on the cyclotene/L-proline mixture (*12*). Application of the Taste Dilution Analysis revealed 16 HPLC fractions, amongst which 12 fractions were judged with TD factors above one (Figure 4).

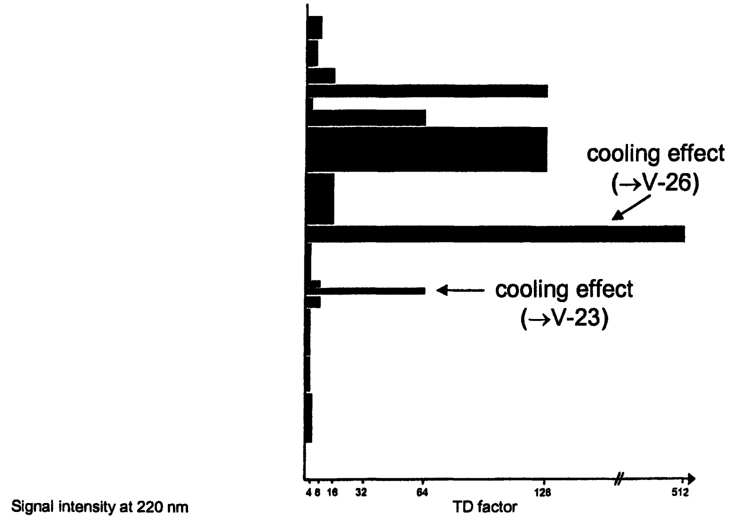

Figure 4. RP-HPLC chromatogram (left side) and Taste Dilution (TD)-chromatogram (right side) of the solvent-extractable fraction of the dry-heated cyclotene/L-proline mixture

The high TD factor of 512 shows the fraction corresponding to V-26 to have by far the highest cooling activity (Figure 4). Whereas the other fractions were judged as bitter tasting, the fraction corresponding to V-23 caused also a cooling sensation, even when the original HPLC fraction was diluted 1:32. Comparing the retention times, the sensory attributes and the mass spectra, the "cooling" compounds in the cyclotene/L-proline mixture were found to be identical to those detected in fractions **V-23** and **V-26** of the glucose/L-proline mixture. Chromatographic separation of the cyclotene/L-proline reaction mixture in combination with HPLC/degustation afforded the "cooling" compounds as pale yellow oils in a purity of more than 99.5%. On the basis of the spectroscopic data obtained by 1D- and 2D-NMR techniques, LC/MS and HRGC/MS the chemical structures of the cooling compounds **V-26** and **V-23** were deduced as 5-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC in Figure 5) and 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC in Figure 5). For a final proof of the proposed structures, both compounds were synthesized in high yields by a hydroxy/amine exchange from 2-hydroxy-3-methyl-2-cyclopenten-1-one and pyrrolidinium acetate (*12*). Although these structures were described earlier to be formed from glucose and L-proline (*5*) as well as from 1-[(2'-carboxy) pyrrolidinyl]-1-deoxy-D-fructose (*15*), their strong "cooling" activity has not been reported earlier.

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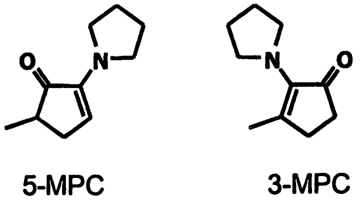

Figure 5. Structures of the "cooling"-active Maillard reaction products 5-methyl-2(1-pyrrolidinyl)-2-cyclopenten-1-one (5-MPC), and 3-methyl-2-(1-pyrrolidinyl)-2-cyclopenten-1-one (3-MPC)

Taking into account that 5-MPC and 3-MPC are generated from the same precursor, the reaction pathways leading to their formation from cyclotene and L-proline were proposed in Figure 6. Imine formation of the amino acid with the carbonyl atoms of the dione formed by de-enolization of the cyclic enolone cyclotene induces a Strecker-type reaction resulting in 3-MPC and 5-MPC.

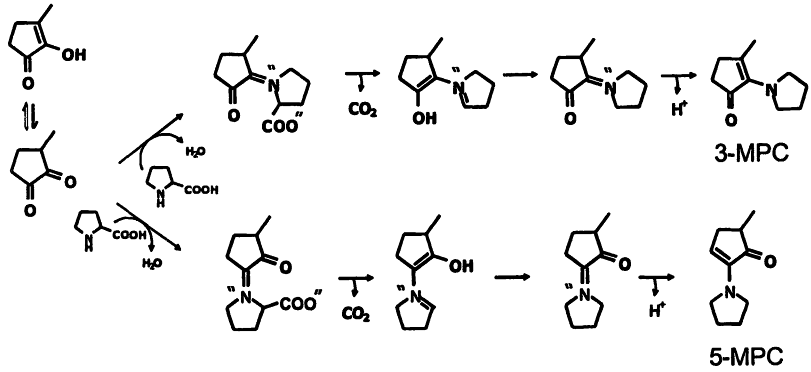

Figure 6. Reaction pathways leading to the formation of 5-MPC and 3-MPC from cyclotene and L-proline

Application of HPLC/TDA on the dry-heated mixture of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone and L-proline (data not shown), generated a reaction product causing the cooling sensation of the HPLC fraction **V-21** (*12*). MS and NMR experiments led to the identification of that "cooling" compound as 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (3(2*H*)-DMPF in Figure 7). As to the best of our knowledge the "cooling" properties of this 3(2*H*)-furanone has been as yet not reported in the literature.

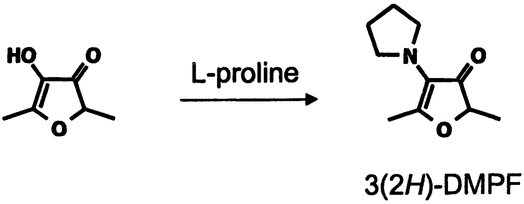

Figure 7. Structure of the "cooling"-active 2,5-dimethyl-4-(1-pyrrolidinyl)-3(2H)-furanone (3(2H)-DMPF)

Structure determination of a pentose-derived “cooling“ compound

In order to check, whether an homologous “cooling” compound might be formed from pentoses via 5-methyl-4-hydroxy-3(2*H*)-furanone (norfuraneol), one of the main pentose dehydration products, a mixture of norfuraneol and L-proline was dry-heated and then analyzed for “cooling” compounds following the analytical strategy detailed above for hexoses. HPLC/degustation discovered a “cooling” compound, the structure of which could be unequivocally identified as 5-methyl-4-(1-pyrrolidinyl)-3(2*H*)-furanone (3(2*H*)-MPF in Figure 8) by MS and NMR measurements as well as synthesis (*13, 16*).

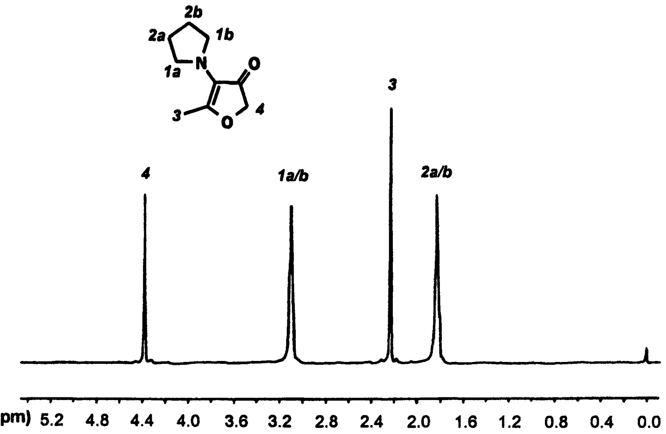

Figure 8. ^{1}H-NMR (360 MHz, $CDCl_3$) of the “cooling”-active 5-methyl-4-(1-pyrrolidinyl)-3(2H)-furanone (3(2H)- MPF)

In addition, 3(2*H*)-MPF could be successfully identified in a roasted mixture of xylose and L-proline by comparing the chromatographic, spectroscopic and sensory data with those of the synthetic reference compound. As to the best of our knowledge the “cooling” properties of the pentose-derived 3(2*H*)-MPF has been as yet not reported in the literature (*16*). On the basis of these results, a reaction pathway leading to the formation of 3(2*H*)-MPF from pentoses and L-proline was proposed in Figure 9 (*13*). Cyclization and water elimination of the 1-deoxypentosone (I), formed by an amino acid assisted degradation of pentoses, affords the 4-hydroxy-5-methyl-2*H*-furan-3-one (II) amongst the major reaction products. In the presence of L-proline, Strecker-type reaction leads to the formation of the corresponding 5-methyl-4-pyrrolidino-3(2*H*)-furanone (3(2*H*)-MPF). In an alternative reaction route, transfer of the pyrrolidine ring onto the 1-deoxypentosone (I) in course of Strecker-type reaction with L-proline might yield the 3(2*H*)-MPF upon ring closure and dehydratization (Figure 9).

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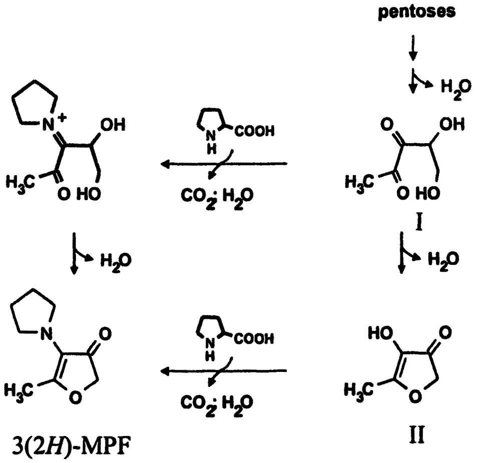

Figure 9. Reaction pathways leading to the formation of the "cooling"-active 5-methyl-4-(1-pyrrolidinyl)-3(2H)-furanone (3(2H)-MPF)

Identification of "cooling" Maillard compounds in dark malts

In order to prove the "naturalness" of these "cooling" Maillard compounds, a solvent-extractable, volatile fraction was isolated from ground malts, and, after preseparation by column chromatography, was analyzed for 3-MPC, 5-MPC, 3(2*H*)-DMPF and 3(2*H*)-MPF by meanse of HRGC/MS (*12*, *16*). As examplified for 3-MPC and 5-MPC in Figure 10, all four "cooling" compounds could be identified in dark roasted malts by comparison of the retention times as well as mass spectra (EI, CI) with those obtained for the synthetic reference compounds. The highest concentrations of the "cooling" compounds were found in caraffa malt (Table III), which is a dark roasted speciality malt, e.g. 101.3 µg 5-MPC were present per kg caraffa malt (*12*). In comparison, a melanoidin malt contained by factors of 4 to 7 lower amounts of these Maillard compounds, e.g. 13.5 µg/kg 5-MPC were determined, which is well in line with the lower roasting degree compared to the caraffa malt. The low degree of Maillard reactions in the light kiln-dried Pilsener malt did not generate the "cooling" compounds in significant amounts.

Table III. Concentrations of "cooling" Maillard compounds in malts

compound	*Concentration (µg/kg) in*		
	Pilsener malt	*Melanoidin malt*	*Caraffa malt*
5-MPC	<0.1	13.5	101.3
3-MPC	<0.1	2.1	9.4
3(2*H*)-DMPF	<0.1	2.2	11.5
3(2*H*)-MPF	<0.1	0.8	4.4

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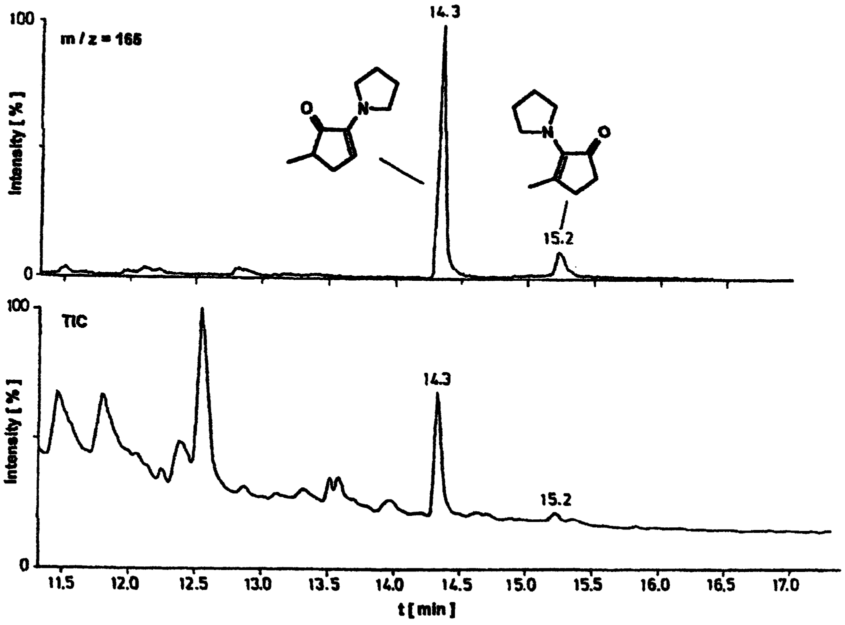

Figure 10. HRGC/MS chromatogram (DB-5) of a fraction isolated from dark malt by column chromatography on Al_2O_3

Sensory properties of "cooling" Maillard compounds

The effect which 3-MPC, 5-MPC, 3(2*H*)-DMPF, and 3(2*H*)-MPF impart to the oral cavity was described by the sensory panel as "cooling" or "fresh". In order to compare the sensory characteristics of these Maillard reaction products with that of (-)-menthol, the most common natural "cooling" agent, the cooling thresholds of 3-MPC, 5-MPC, 3(2*H*)-DMPF, and 3(2*H*)-MPF were determined in water by using a triangle test (Table IV). 3(2*H*)-MPF was evaluated with the lowest detection threshold concentration causing a pronounced oral cooling sensation at the very low level of 1.5-3.0 mg/kg water (*16*). Also the cyclotene-derived compounds 3-MPC and 5-MPC exhibited a pleasant, long-lasting cooling effect in the mouth (*12*). 3(2*H*)-DMPF was evaluated with the highest "cooling" detection theshold at the relatively high level of 100-140 mg/kg. In comparison to 3(2*H*)-DMPF, the 5-MPC and 3(2*H*)-MPF were found to have 18- or 53-fold lower "cooling" thresholds, respectively, and these were only higher by factors of 5 or 1.5, respectively, than the threshold concentration determined for (-)-menthol (*16*).

Because all the compounds investigated are volatile, we, in also were interested in their odor thresholds. The lowest odor threshold concentrations with 0.1-0.2 mg/kg was found for (-)-menthol eliciting a strong mint-like odor. In contrast, the "cooling" Maillard compound 3(2*H*)-MPF did not show any odor, and 5-MPC, 3-MPC and 3(2*H*)-DMPF were evaluated only with very high odor thresholds (Table IV). Calculating the ratio of cooling threshold to odor threshold clearly demonstrated a value below one for 3(2*H*)-MPF and 3-MPC, exhibiting no or only a very faint odor, respectively (*16*). In comparison, the ratio for (-)-menthol is 9.5, i.e., the odor threshold is well below the cooling threshold, implying that menthol cannot be used to provide cooling effects to food applications without imparting a predominant mint-like odor, which would be difficult to mask. In contrast, natural "cooling" Maillard compounds, in particular 3(2*H*)-MPF and 3-MPC, offer new possibilities of imparting a pleasant "freshness" and "cooling" effect to the oral cavity during consumption of non-mint food compositions, such as, confectionery products, malted beverages or fruity flavorings.

Table IV. Comparison of oral "cooling" and retronasal odor thresholds of Maillard compounds and (-)-menthol

compound	*threshold conc. [mg/kg]*[a]		*odor quality*	*Ratio (cooling/ odor)*
	cooling	*odor*		
3(2*H*)-DMPF	100.0 – 140.0	30.0 – 60.0	nutty, roasty	2.7
3-MPC	29.0 – 43.5	43.5 – 72.5	faintly amine-like	0.8
5-MPC	4.5 – 9.0	2.6 – 5.2	faintly mint-like	1.7
3(2*H*)-MPF	1.5 – 3.0	---	*odorless*	<0.01
(-)-Menthol	0.95 – 1.85	0.10 – 0.20	strongly mint-like	9.5

[a] Threshold concentrations were determined in tap water by using a triangle test (*12*).

In order to gain insights into the dynamics of the "cooling" perception, time/intensity courses were determined for the Maillard compounds, 3(2*H*)-MPF, 3-MPC and 5-MPC, and for (-)-menthol. Aqueous solutions containing these compounds in concentrations of 50-fold above the corresponding "cooling" threshold were presented to a sensory panel, which was asked to evaluate the intensity of the cooling effect perceived in the oral cavity on a scale from 0 (absent) to 5 (strong) over a period of 30 min (Figure 11).

These solutions were chewed for exactly 60 sec, whilst the intensity of the cooling sensation was determined each 10 s. After 1 min of chewing, the material was spat out, and the "cooling" intensity in the after-taste was evaluated after an additional 2, 3, 5, 10, 15, 20, 25, and 30 min. For the Maillard compounds 3-MPC (■ in Figure 11) and 5-MPC (● in Figure 11), and, in particular, for 3(2*H*)-MPF (▲ in Figure 11), an intense cooling effect was perceived instantaneously after taking up the solutions into the oral cavity. Chewing the material led to an increase of the intensity within 30 s from about 1.5 to 4.5 for 3- and 5-MPC, and from 3 to 5 for the 3(2*H*)-MPF, after which the cooling sensation remained constant in intensity (Figure 11). After spitting out the material, the intensity diminished by 50% after 5-10 min. For both the MPCs, a significant cooling effect was still perceivable after 20 min, whereas the 3(2*H*)-MPF caused a significant cooling sensation even after 30 min. In comparison, sensory evaluation of the aqueous solution of (-)-menthol (—— in Figure 11) revealed a significant cooling sensation at first after 10 sec. After 30 s, the (-)-menthol also caused the maximum cooling effect, but with somewhat lower intensity compared

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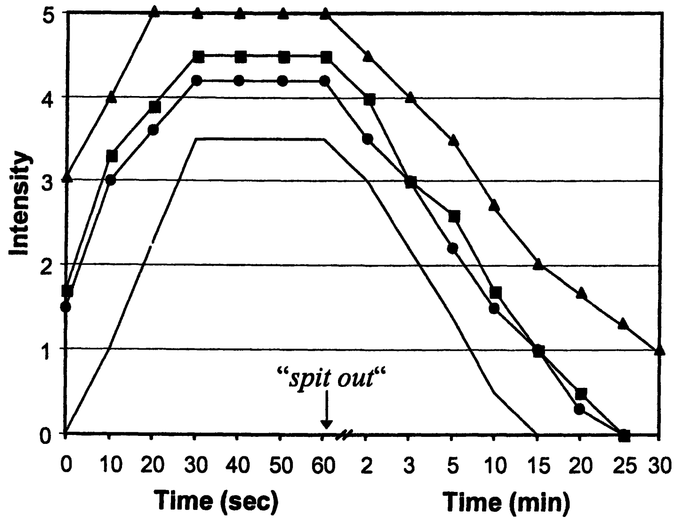

Figure 11. Time/intensity-course of oral "cooling" sensation induced by (-)-menthol (——), 3-MPC (—■—), 5-MPC (—●—) and 3(2H)-MPF (—▲—).

to the Maillard-type coolants. After spitting out the solution, the intensity of the cooling effect diminished again and was still perceivable after 10 min. However, after 15 min, a significant "cooling" effect could no longer be detected. These data indicate that the "cooling" effect provided by the Maillard compounds starts more rapidly and is significantly longer lasting than that of (-)-menthol (*12*).

Conclusions

A novel bioassay, the Taste Dilution Analysis (TDA) has been demonstrated as a activity-guided screening technique enabling a straightforward localization of sapid tongue-affecting compounds in complex mixtures of compounds present in Maillard reaction mixtures or foods. Using this novel analytical strategy, useful informations can be obtained which might be helpful to extend the knowledge on taste-active compounds generated during food processing, e.g. during kiln-drying of malt. In addition, the results of this study indicate that candidates of the multiplicity of Maillard reaction products, in particular, 3(2*H*)-MPF, 3-MPC, and 5-MPC, might be used to evoke certain "cooling" effects during consumption of non-mint food compositions such as, drinking water, confectionary products, malted and citrus beverages as well as fruity or browned flavors.

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Chapter 26

Identification of Maillard-Type Aroma Compounds in Winelike Model Systems of Cysteine–Carbonyls: Occurrence in Wine

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The role of carbonyl (acetoin and acetol) and dicarbonyl (glyoxal, methylglyoxal, diacetyl and pentan-2,3-dione) compounds associated with amino acids in the formation of Maillard-type odorous products under conditions close to those of wine, i.e. low pH, aqueous medium and low temperatures is described. Many carbonyl and α-dicarbonyl compounds were present in wine and their origins were studied in our laboratory and their contents specified. Among the amino acids present in wine, cysteine is most remarkable. In a first step, cysteine was quantified by using derivatization-HPLC-fluorimetry and the parameters of cysteine formation in wine were studied. Then, reaction products between cysteine and carbonyls, mainly heterocyclic compounds such as thiazole, 4-methylthiazole, 2,4-dimethylthiazole, 2-acetylthiazole, 2-acetylthiazoline, trimethyloxazole, 2-methyl-3-furanthiol, 2-furanmethanethiol and thiophene-2-thiol were identified. The descriptors are "very ripe fruit" for trimethyloxazole; "popcorn", "roasted", "peanuts" for thiazoles and "roasted coffee" or "burnt rubber" for furans and thiophenes. Some of these molecules were identified for the first time in wine. To determine the concentrations of all these compounds, quantitative assay methods were optimized using GC/MS for identification, GC/FPD for sulfur compounds, GC/NPD for nitrogenous compounds and derivatization-HPLC for carbonyl compounds. Also, attempt was made to detect these Maillard-type compounds in wines from various origins.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

An important trust of our work is to define the role of lactic acid bacteria in wine aroma. In particular, we are interested (1) in the flavor of products synthesized by bacteria, and (2) in the formation of aroma by reactions between amino acids and carbonyl compounds later during the aging period of wine. Thus, an overview of the origin and evolution of carbonyl compounds in wine is presented.

Moreover, we are interested in cysteine in wine, an amino acid present in small quantities but playing a well-known role in the reaction with carbonyl compounds. Thus, results of cysteine levels in wine are first presented, before describing a study of the products of reaction between amino acids and α-dicarbonyl compounds under mild conditions: ethanol/water 12%, a pH close to that of wine and temperature of 20 °C (a very low temperature in comparison to conditions in the Maillard reaction system). In this way, the heterocyclic compounds found in model solutions were quantified in wine.

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Experimental

Materials. Cysteine, diacetyl, pentan-2,3-dione, methylglyoxal, acetoin, acetol, butan-2,3-diol, propan-1,2-diol and compounds used for identification of final products (thiazole, 2-acetylthiazole and 2-acetyl-2-thiazoline) were purchased from Sigma Aldrich Chemical Co. (Fallavier, France) Inorganic reagents and solvents were all commercial products of analytical grade. Each cysteine / methylglyoxal mixture was prepared in stoichiometric conditions (20 mM, 10 mM, 5 mM, 0.4 mM, 0.2 mM and 0.1 mM) in an hydroalcoholic solution (12% vol.) and adjusted to pH 3.5 with 1N HCl, or 1N NaOH or KOH (*1*). The solutions were stored at 25 °C in the dark during various storage periods.

Cysteine and heterocycle solutions (thiazole, 2-acetylthiazole, acetylthiazoline) were prepared at 20 mM and analyzed during a 3-week storage period. The compound 2-sulfanylethanal was synthesized by equimolar reaction (15 mM) between H_2S and glyoxal in ethyl acetate-like solvent. It was then derivatized by O-(2,3,4,5,6-pentafluorobenzyle)hydroxylamine hydrochloride (PFBOA) (Aldrich) to form an oxime that was injected onto a chromatographic column coupled with different detectors (FID, NPD, FPD and MS).

The carbonyl compounds were determined by direct injection or after derivatization (*2*). Hydroxyketones were quantified with a GC-FID system and a capillary Carbowax column (Biochrom, Vindelle, France). All dicarbonyl compounds were quantified in gas chromatography after derivatization. We used pentafluorobenzyl hydroxylamine hydrochloride derivatives and diaminobenzene derivatives. The latter method has proved more efficient (*3*). Good specificity was obtained with dicarbonyls and these could be determined without previous solvent extraction. Quinoxalines were detected at 313 nm. DAB derivatives could be injected after extraction with dichloromethane into a gas chromatograph and detected by mass spectrometry with selected ions.

An HPLC method for the determination of free amino acids and especially that of cysteine in wine was reported by Pripis-Nicolau *et al.* (*4*). This technique involves the use of a pre-column derivatization with iodoacetic acid and OPA.

GC/FPD Analysis. Determination of the heterocycles (thiazole, 2-acetylthyazole, 2-acetyl2-thiazoline) and N-(2-sulfanylethyl)-2-oxopropanamide (SOPA) was performed on a gas chromatograph (Agilent Tech. 6890, Massy, France) coupled with a flame photometric detector (FPD). Thiazol-2-carboxaldehyde was used as an internal standard. The column was a HP 5 (30 m × 0.53 mm × 5 μm) (Agilent Technologie, Massy, France). The oven temperature was kept at 50 °C for 1 min, programmed at a rate of 2 °C/min to 150 °C, then at a rate of 10 °C/min to 200 °C. Two millilieters of the headspace were injected. Light sulfurous compounds were determined by a second gas chromatograph (Agilent Tech. 5890) coupled with an FPD. Thiophene was used as an internal standard. An HP 5 column (30 m × 0.53 mm × 5 μm) was used. The oven temperature was kept at 32 °C for 1 min, programmed at a rate of 10 °C/min to 100 °C, then at a rate of 20 °C/min to 180 °C, with the final step lasting 20 min.

GC/NPD Analysis. After derivatization with PFBOA 2-Sulfanylethanal could be determined with an NPD under the same chromatographic conditions.

GC/FID Analysis. Acetaldehyde analysis was performed by gas chromatography (Agilent Tech. 5890). The internal standard used was 4-methylpentan-2-ol. The column was a capillary CP Wax 57 CB (50 m x 0.25 mm x 0.2 μm) (Biochrom, Vindelle, France). The oven temperature was kept at 40°C for 5 min and programmed at a rate of 4 °C/min to 200°C; split injection at a flow of 60 ml/min.

GC/MS Analysis. A first gas chromatograph (Agilent Tech. 5890) was coupled with a mass spectrometer (Agilent Tech. 5972A, electronic impact: 70 eV, eMV: 2.7 kV). A BP 21 polar column (50 m x 0.25 mm x 0.20 μm) (SGE Sorl, Georges, France) was used to identify 2-sulfanylethanal . The oven temperature was programmed from 50 °C to 220 °C at a rate of 3°C/min, the initial step lasting 1 min and the final step lasting 40 min. The carrier gas was helium (1.6 mL/min). The injector was a splitless system: the splitless time was 20 s and split vent was 30 mL/min. For other heterocyclic compounds, a second gas chromatograph (Agilent Tech. 6890) was coupled with a mass spectrometer (Agilent Tech. 5972A, electronic impact: 70 eV, eMV : 2.7 kV). The column was a CP-sil 5CB (50 m × 0.32 mm × 0.12 μm) (Varian Chrompack France, Les Ullis, France). The oven temperature was programmed from 50 °C to 220 °C, the initial step lasting 1 min, at a rate of 2 °C/min to 150 °C, then a rate of 5 °C/min to 220 °C and with a final step lasting 30 min. The carrier gas was

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helium (1.7 ml/min). The injector was a splitless system. Quantitative determination of 2-acetylthiazole, 2-acetyl 2-thiazoline SOPA was done in SIM mode selecting ions of m/z = 127 for 2-acetylthiazole, m/z = 129 for 2-acetyl 2-thiazoline, m/z = 104 for SOPA and m/z = 113 for the internal standard (thiazole carboxaldehyde).

Results and Discussion

The α-dicarbonyls in wine were glyoxal, methylglyoxal, diacetyl, and pentane-2,3-dione. Glyoxal was encountered in large quantities only in wine made from botrytized grapes (like Sauternes), while the other compounds were synthesized during alcoholic fermentation by yeast. Diacetyl and methylglyoxal were produced by bacteria in malolactic fermentation. These compounds could be reduced to hydroxyketones, i.e. acetoin, acetol and 3-hydroxypentan-2-one. In wine, hydroxyketones were then reduced to diols, i.e. butan-2,3-diol, propan-1,2-diol and pentan-2,3-diol.

In red wine, α-dicarbonyls were found at variable levels (concentrations in white wines were always lower). Diacetyl concentrations ranged from 1 to 4 mg/L. Methylglyoxal was between 0.04 and 0.5 mg/L after alcoholic fermentation and pentanedione between 1 and 2 mg/L during fermentation. However, in finished wine the levels were less than 0.5 mg/L. During malolactic fermentation the levels of diacetyl and methylglyoxal increased and bacteria produced diacetyl between 3 and 8 mg/L. Diacetyl and other dicarbonyls decreased during fermentation to form hydroxyketones. Acetoin and acetol occurred at about 20 mg/L and hydroxypentanone at a maximum of about 3 mg/L.

The evolution of α-dicarbonyls in wine was quite different between diacetyl and pentanedione due to the bacterial activity. Pentanedione was synthesized during alcoholic fermentation by yeast and later decreased. Its level in finished wine was low. On the other hand, diacetyl was synthesized by yeast and by bacteria and its level was higher. This is important for wine aroma whenever malolactic fermentation occurs. The dicarbonyl compounds in wine have a system of reduction. The reduction of vicinal dicarbonyls leads to a concomitant reduction in flavor intensity (Figure 1).

The sensorial impact of α-dicarbonyl compounds is great. The odor of diacetyl is butter-like, while the aroma of methylglyoxal is lighter and fresher but is similar to diacetyl. Pentanedione is fatty but also has a strong toasted odor. Of all the compounds detected, only diacetyl was found in wine close to the odor threshold. The α-dicarbonyls have an incidence in wine technology, because they can combine with sulfur dioxide. In this case, they lose their aroma but the phenomenon is reversible when the SO_2 disappears.

After fermentation, dicarbonyls could be of interest in the aroma of aging wine so research was focused on the reactivity of these compounds with amino

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acids. The first results showed that dicarbonyl could react with amino acids in the conditions of wine and could produce very interesting aromas, e.g. not only with valine, leucine, isoleucine and phenylalanine but also with methionine and cysteine. Because cysteine is an amino acid which has not been widely studied in wine, we have recently developed an HPLC method allowing the determination of all amino acids in wine (except proline) and their evolution during fermentations. Cysteine increased during alcoholic fermentation like ornithine, while other amino acids were consumed by yeast by about 90%. Cysteine levels in wine were low with a mean between 1 and 2 mg/L.

For model solutions between dicarbonyls and cysteine or other amino acids, we prepared 20 mmoles of each one and analyzed the aroma by tasting or by GC/sniffing and volatile compounds, by GC/MS and also with various detectors (FID or specific FPD and NPD). Many odorous heterocycles like thiazole, acetylthiazole, alkyloxazole, thiophene and furane were detected (Figure 2). These compounds are well known products of the Maillard reaction between sugars and amino acids. This non-enzymatic reaction produces a number of aromatic compounds like HMF and furfural. It also produces not only small reactive molecules like hydrogen sulfide, ammonia and acetaldehyde by Strecker degradation of amino acids in the presence of dicarbonyl compounds, but also specific aldehydes and heterocyclic compounds. However, the reaction in wine is very different, since wine is not heated and the temperature is between 10 and 35 °C. Normally the wine is dry but we have seen wines which contain some α-dicarbonyls.

Under the conditions of pH and temperature experienced in wine, many products, including specific aldehydes were found with amino acids such as valine, leucine, isoleucine, phenylalanine and methionine (*1*). With cysteine, no specific labile aldehydes were found, but many heterocycles were present. In addition, H_2S, methanethiol, carbon disulfide, alkylthiazole, alkylpyrazine, alkyloxazole; 2-acetyl-2-thiazoline and 2-acetylthiazole were detected.

In these heterocyclic compounds, the influence of pH and temperature on the degradation of cysteine and the formation of 2-acetylthiazole was investigated. Cysteine degradation was observed with three carbonyl compounds, namely acetoin, diacetyl and methylglyoxal. The decomposition of cysteine was faster at pH 8.0 than at pH 3.5 and was particularly slow in the presence of acetoin. With regard to the reaction products, higher amounts of acetylthiazole and 2-acetyl-2-thiazoline were formed at the lower pH. At pH 3.5 the solution released popcorn, roasted hazelnuts, while at pH 8, we detected rotten egg, cabbage and roasted meat notes. Therefore, an acid pH close to that of wine is more favorable for the formation of acetylthiazole and acetylthiazoline. No large differences were observed in terms of olfactory nuance between 10 and 40 °C, nor in terms of the compounds identified in the solution.

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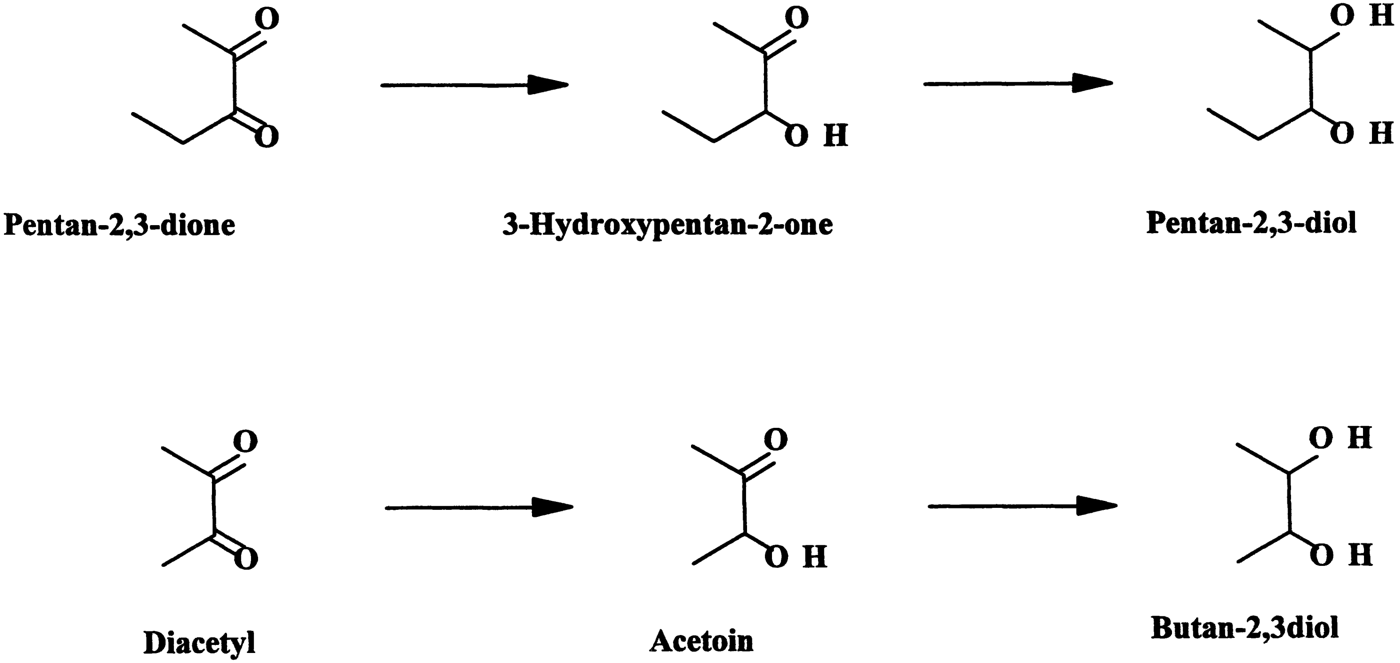
O
O
O
O H
O H
O H
Pentan-2,3-dione
3-Hydroxypentan-2-one
Pentan-2,3-diol
O
O
O
O H
O H
O H
Diacetyl
Acetoin
Butan-2,3diol

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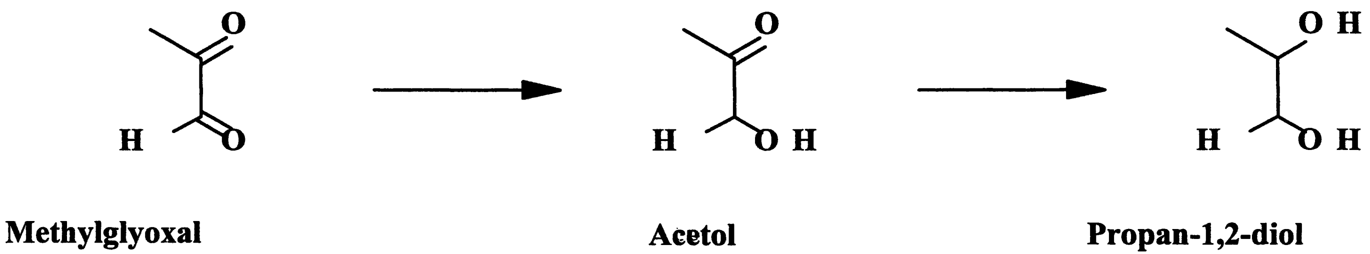
O
H
O
Methylglyoxal
O
H
O H
Acetol
O H
H
O H
Propan-1,2-diol

Figure 1. Redox system of the different dicarbonyl and related compounds in wine.

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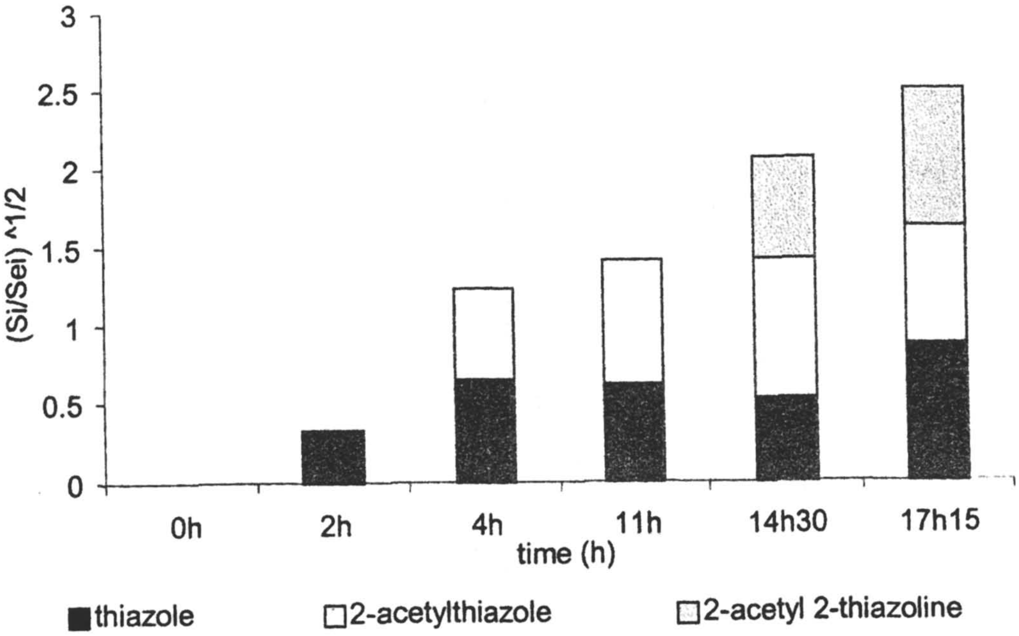

Figure 3: Heterocyle evolution in cysteine / methylglyoxal solution (0.1 mM).

Figure 2. Heterocyclic compounds identified in cysteine/dicarbonyl solutions.

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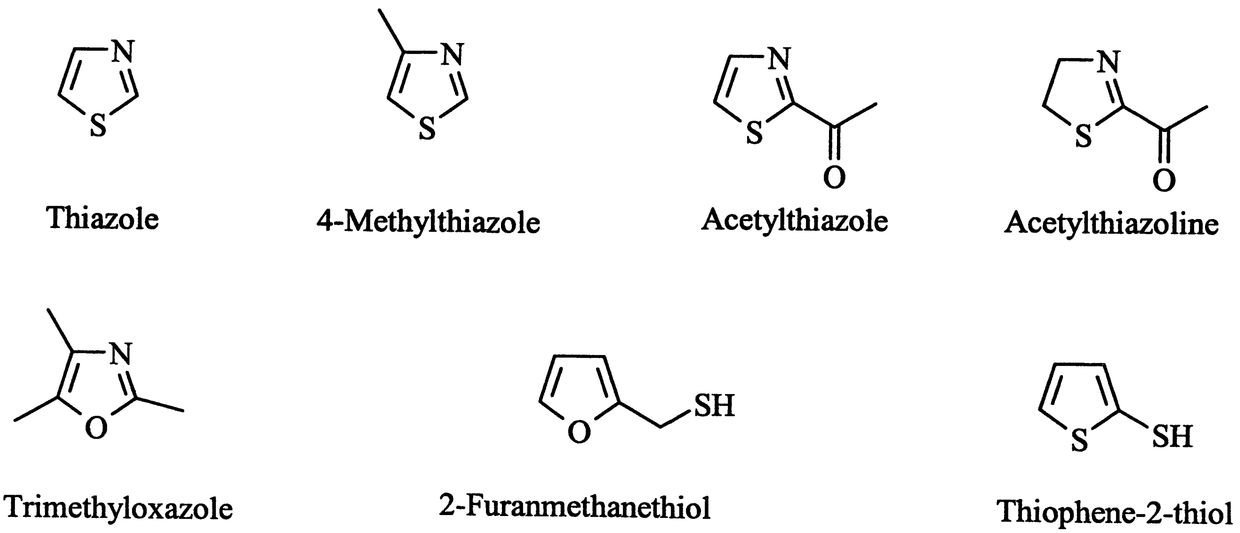
Thiazole
4-Methylthiazole
Acetylthiazole
Acetylthiazoline
Trimethyloxazole
2-Furanmethanethiol
Thiophene-2-thiol

Figure 3. Heterocyle evolution in cysteine / methylglyoxal solution (0.1 mM).

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Table I. Thiazoles in wine.

	thiazole	*4-methiazole*	*2,4-dimethiazole*	*2-acthiazole*	*2-acthiazoline*
St Emilion	(6 samples)				
Average (μg/L)	4	0	1	1	3
Frequency (%)	100	0	17	67	50
Médoc	(6 samples)				
Average (μg/L)	5	1	4	2	5
Frequency (%)	67	33	83	100	100
Champagne	(7 samples)				
Average (μg/L)	5	2	4	1	6
Frequency (%)	86	43	71	71	57
Wines for Champagne	(3 samples)				
Average (μg/L)	3	0	3	1	6
Frequency (%)	67	0	33	33	67
Languedoc red wines	(7 samples)				
Average (μg/L)	4	0	1	0	1
Frequency (%)	100	0	29	29	29

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During the degradation of cysteine at 2 mmole and at 10 °C, cysteine was found to be degraded; at the same time, 2-acetyl-2-thiazoline increased with a maximum after 3 h, while 2-acetylthiazole increased regularly. Acetylthiazoline presented a characteristic curve of an intermediate reaction (*1*). This molecule may be oxidized in acetylthiazole. A mechanism was proposed by Griffith and Hammond (*5*), where 2-acetylthiazoline served the intermediate during the formation of acetylthiazole from cysteine and methylglyoxal. The reaction was conducted at low temperature but at pH 8. Moreover, thiazole and acetylthiazole were found in the solution of methylglyoxal and cysteine after 5 min, but no acetylthiazoline if cysteine and methylglyoxal were diluted to 0.4 mM (*6*). In this case acetylthiazoline appeared only after 14½ h (Figure 3). In our lab, we have determined an unknown compound which is present during the formation of acetylthiazole from 2-acetylthiazoline, and this compound has also been determined in the solutions of cysteine and methylglyoxal which was termed N-(2-sulfanylethyl)-2-oxopropanamide or SOPA (*7,8*). It is now necessary to confirm that under mild conditions how thiazole, acetylthiazole and acetylthiazoline are synthesized and the role of the Schiff base or SOPA in these formations.

The initial results on heterocycles found in wine are shown in Table I; thiazole, 4-methylthiazole, 2,4-dimethylthiazole, 2-acetylthiazole and 2-acetyl-2-thiazoline have now been determined. All these heterocycles are found in wine, except for some wines in which 4-methylthiazole and 2-acetylthiazole are present. Champagne and Medoc wines have been found to have the highest concentrations. Only 2-acetyl-2-thiazoline has levels close to its aroma threshold.

Acknowledgment

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Chapter 27

Aroma Components of Wines from Chardonel: A French–American Hybrid Grape

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Chardonel, a Chardonnay x Seyval hybrid, is a wine grape varietal suitable for Midwestern U.S. production of Chardonnay style wines. Volatile components of three Chardonel wines and one Chardonnay wine produced in Illinois were isolated by liquid-liquid continous extraction (LLCE) and headspace-solid phase microextraction (H-SPME). Characteristic aroma components were identified by gas chromatography-olfactometry (GCO) methods, including aroma extract dilution analysis (AEDA), frequency of detection (FDT) and intensity scaling. Quantification was performed by H-SPME-GC-MS. Various esters, β-damascenone, 3-methyl-1-butanol and 2-phenylethanol were found to be characteristic components of Chardonel wine.

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Wine production in the United States has traditionally taken place in the Napa Valley region of California and in the Finger Lakes region of New York. In the last decade there has been increased interest in wine production in other regions throughout the U.S. including the Midwest. Chardonel, a Chardonnay x Seyval hybrid, is a relatively new variety released by the New York State Agricultural Experiment Station (*1*). This is a late-ripening white grape that withstands cold weather better than Chardonnay. For this reason Chardonel has become a popular variety amongst Midwestern winegrowers. Furthermore, Chardonel makes excellent dry, semidry and sparkling Chardonnay style wines having lightly fruity and delicate flavors (*2*). Chardonel wines are particularly suitable for oak aging.

The flavor of wine is highly variable and depends on many factors including grape variety, as well as agronomic, fermentation and storage conditions. The aroma components of wines include alcohols, esters, acids, terpenes, and other volatile compounds. Numerous sample preparation techniques including solvent extraction (*3-10*), solid phase extraction (*6,8,11*), headspace analysis (*5*) and other miscellaneous methods (*6,7*) such as direct injection (*7*) have been widely used in the gas chromatographic analysis of wine volatile constituents. Due to its high sensitivity, simplicity and ease of use, headspace-solid-phase microextraction (H-SPME) has been extensively applied in recent years for the analysis of wine volatiles (*11-17*).

Gas chromatography-olfactometry (GCO) dilution techniques, including aroma extract dilution analysis (*5,8*) and CharmAnalysis™ (*18*), and scaling techniques, such as frequency of detection (FDT)(*9*) and intensity estimation (*9,10*), have been employed for the analysis and characterization of aroma-active components of wines. In AEDA, serial dilutions of a flavor extract are evaluated by GCO to provide flavor dilution (FD) factors for the aroma-active components. An FD-factor is reflective of the odor-activity value (concentration/odor detection threshold) for a compound and can be used to gauge its relative aroma contribution. In addition to the GCO-dilution techniques, it is possible to assess the volatile components isolated with H-SPME and GCO using odor intensity scaling techniques (*19*).

The purpose of the present study was to identify and characterize the aroma-active components of Chardonel wines produced in Illinois.

Experimental

Materials

Wines were obtained from commercial wineries within Illinois (Table I). Reference standards and other reagent grade chemicals were obtained from Aldrich Chemical Co. (St. Louis, MO), except for β-damascenone which was a gift from Firmenich Co. (Princeton, NJ). Dichloromethane (Aldrich) was redistilled prior to use. Deodorized-distilled water was prepared by boiling distilled water in an open flask until its volume was decreased by two-thirds.

Table I. Composition of Chardonel wines

Wine / vintage (origin)	*Code*	*TA (g/L)*[a]	*pH*	*EtOH (%v/v)*	*VA (g/L)*[b]
Chardonel 1999 (Whittington, IL)	CL1	6.9	3.3	11.0	0.036
Chardonel 1999 (Greenup, IL)	CL2	7.2	3.3	11.6	0.048
Chardonel 1999 (Coden, IL)	CL3	6.8	3.5	11.5	0.072
Chardonney 1999 (Mt.Vernon, IL)	CY1	6.3	3.4	11.2	0.054

[a]Titratable acidity expressed as tartaric acid. [b]Volatile acidity expressed as acetic acid.

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Analytical Procedures

Wine Compositional Analysis

Titratable acidity, volatile acidity, pH, sulfur dioxide, residual sugar, and ethanol (by ebulliometric analysis) were measured using published procedures (*20*).

Sensory Evaluation

Aroma profiling was conducted by a five member panel familiar with wine judging and sensory evaluation procedures. Intensities of eight aroma terms (fruit, vegetal, floral, dried fruit, spicy, butter, oak, and complexity) were rated using a published procedure (*21*). Aroma profiles reported herein are based on consensus ratings by the panel.

Liquid-Liquid Continuous Extraction (LLCE) and Class Fractionation

Volatile compounds were extracted using a liquid-liquid continuous extractor according to Baek *et al.* (*3*) with some modifications. Wine sample (250 mL) plus 900 mL of deodorized-distilled water and 10 μL of an internal standard solution (10 mg/mL of 2-methyl-3-heptanone and 10 mg/mL of 2-ethyl butanoic acid in methanol) were continuously extracted with dichloromethane (200 mL) for 18 h at room temperature (~23°C). Excess water was removed from the extract by freezing out and then by drying over 10 g of anhydrous sodium sulfate. Extract was concentrated to 50 mL on a Vigreux column in a 48°C water bath. High vacuum distillation (~5 x 10-5 Torr) clean-up step was used to remove nonvolatile materials from the extract (*22*). The distillate was then subjected to compound class fractionation for separation of acidic volatiles from neutral/basic compounds as previously described (*22*). Each extract fraction was concentrated to 500 μL and stored at -20 °C until analyzed.

Gas chromatography-Olfactometry (GCO) and Aroma Extract Dilution Analysis (AEDA)

General procedures for AEDA have been previously described (*23*). Serial dilutions (1:3, 1:9, etc.) of each fraction from the CL3 sample extracts were prepared in dichloromethane. Each dilution was transferred to a 2 ml vial equipped with a teflon-lined screw cap and stored at –20°C until analyzed. GCO was conducted on an HP6890 GC (Agilent Technologies, Inc.) equipped with a flame ionization detector and sniff port (DATU Technologies, Geneva, NY). Separations were performed on a DB^{TM} –FFAP (15 m length x 0.32 mm i.d. x 0.25 μm film thickness; J&W Scientific, Folsom, CA) capillary column. In order to minimize sample decomposition, each dilution (1 μL) was injected in the cool (38°C) on-column mode. Column effluent was split 1:1 between FID and sniff port using deactivated fused silica tubing (1-m length x 0.25 mm i.d.). FID and sniff port interface were maintained at 250°C. Helium was used as a carrier gas at 1.2 mL/min. GC oven temperature was programmed from 35°C to 225°C at a rate of 10°C/min with initial and final hold times of 5 and 20 min, respectively. GCO was performed by two experienced panelists. AEDA results are expressed as arithmetic means of $\log_3$FD factors, where the FD (flavor dilution) factor represents the highest dilution at which an odorant was last detected during GCO.

In addition to AEDA, GCO was performed on CDL1, CDL2 and CDL3 sample extracts by four panelists and data recorded as frequency counts (i.e. frequency of detection, FDT = number of times detected out of four attempts)(*9*).

Headspace-Solid Phase Microextraction-(H-SPME)-GCO

Wine sample (5 mL), 2 g of NaCl, and a teflon-coated magnetic stir bar were placed in a 22-mL vial and the vial sealed with a Teflon-lined silicon septum. The sample was stirred for 20 min at room temperature (~23°C) and then a SPME fiber (50/30 μm DVB/Carboxen^{TM}/PDMS or 85 μm polyacrylate fiber; Supelco, Bellefonte, PA) was exposed to the sample headspace for an additional 10 min while stirring. After sampling, the SPME fiber was desorbed by splitless injection (injector temperature, 260°C; splitless time, 4 min; vent flow, 50 mL/min) into the GCO system described earlier. GCO was performed by three experienced panelists who rated odor intensity on an 8-point scale (where 0 = no odor and 7 = very strong odor).

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Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS system consisted of an HP 6890 GC/5973 mass selective detector (MSD; Agilent Technologies, Inc.). Solvent extracts (1 µL) were injected in the cool (38°C) on-column mode. For analysis by H-SPME, fiber was desorbed by splitless injection as described above. Separations were performed on a DB™-FFAP column (30 m length x 0.25 mm i.d. x 0.5 µm film thickness; J&W Scientific). Helium was the carrier gas at a constant flow of 1.0 mL/min. GC oven temperature was programmed from 40°C to 225°C at a rate of 4°C/min with initial and final hold times of 5 and 30 min, respectively. MSD conditions were as follows: capillary direct interface, 250°C; ionization energy, 70 eV; mass range, 35-300 a.m.u.; EM voltage (Stune + 200 V); scan rate, 5.27 scans/s.

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Qualitative and Quantitative Analyses

Compound identification was based on comparison of GC retention indices, mass spectra, and aroma properties of unknowns with those of authentic reference compounds analyzed under identical experimental condition as mentioned above. Selected compounds were quantified by H-SPME and internal standard methodology. Wine sample (50.0 mL) was spiked with multiple internal standards (methyl pentanoate, 2.31 µg; 3-heptanol, 1.93 µg; 2-ethylbutanoic acid, 2.31 µg; 6-undecanone, 2.27 µg; 2-undecanol, 1.92 µg). Five milliliters of this solution plus 2 g of NaCl were transferred to a 22-mL vial and sealed with a teflon-lined silicon septum. Automated H-SPME analyses were conducted on a MPS2 multipurpose sampler (Gerstel, Germany) using a DVB/Carboxen™/PDMS fiber as described above except that sample preincubation temperature was 35°C (20 min) and fiber exposure time was 20 min. Sample agitation was provided by the MPS2 during preincubation and fiber exposure. Calibration curves were generated in a wine mimic matrix consisting of 11% (v/v) ethanol, 7.0 g/L tartaric acid and 1.0 g/L glucose in deodorized-distilled water.

Results and Discussion

Composition and Sensory Properties

For this study three Chardonel wines and one Chardonnay wine produced in Southern Illinois were selected. The compositional parameters of these wines (Table I), including pH, titratable acidity and ethanol, were consistent with literature values reported previously for Chardonnay style wines (*21*). Volatile acidity levels for all wines were well below legal tolerance levels (*24*).

All wines were subjected to sensory descriptive analysis in which eight aroma notes were rated by an expert panel (Figure 1). Fruit, floral, dried fruit and spicy notes were predominant in all four wines. Likewise, aroma complexity was rated

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similarly high for all wines. Butter aroma was detected in CL2 only. Vegetal aroma was scored highest in CL3, while strong oak and spicy aroma notes were detected in CL1. CL1 was regarded by the panel as having a 'Brett' (*Brettanomyces*) flavor defect. Results of volatile analyses revealed the presence in CL1 of a relatively high level of 4-ethylphenol, which is a known indicator compound of 'Brett' flavor (*18*). The pronounced vegetal note of CL3 is believed to be due to the presence of a relatively high level of methional in this wine (measured by H-SPME-GCO analysis). In the literature vegetal flavor is generally considered to be the consequence of methoxypyrazine compounds, e.g. 3-isobutyl-2-methoxypyrazine (*25*). However, in the present study no methoxypyrazine compounds were detected by either GCO or GC-MS in any of the four wine samples examined.

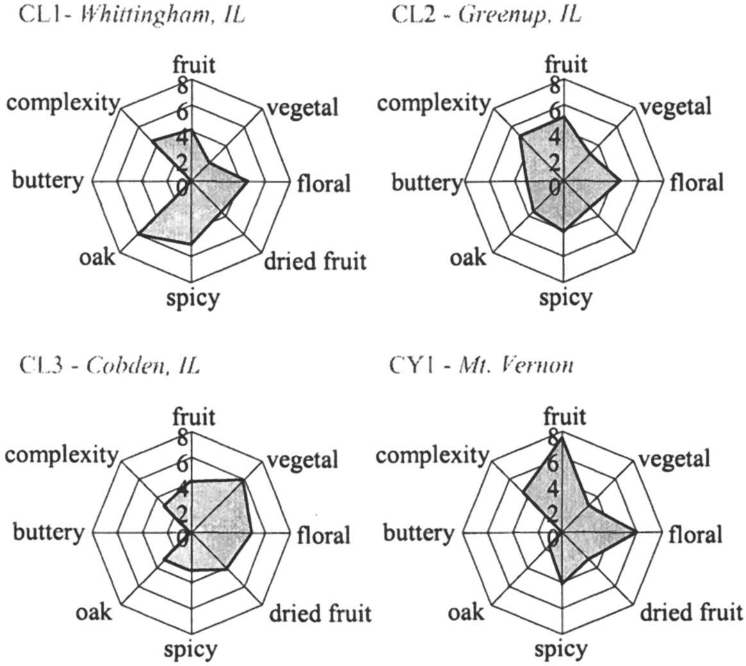

Figure 1. Flavor profile comparison of Chardonel and Chardonnay wines

GCO-FDT and AEDA

Aroma extracts were prepared from each of the three Chardonel wines (CL1, CL2 and CL3) by liquid-liquid continuous extraction (LLCE) and high vacuum distillation. Extracts were then subjected to GCO-FDT to compare the major aroma-active components of these wines (Table II). A total of 23 odorants were detected in these wines. Each of the seven aroma components of the acidic fraction was detected at a similar frequency in all three wines. The wines shared many of the same neutral/basic aroma components, but differed in some important distinguishing ones. For example, the 'Brett' flavor character of CL1 may have been due to the presence of guaiacol (**18**, *smoky*) and 4-ethylphenol (**20**, *phenolic*), which were not detected in either CL2 or CL3. An unknown compound with a *yeasty* aroma note was also detected at a higher intensity in CL1 than CL2 and CL3. Likewise, the butter character detected in CL2 was likely caused by the presence of 2,3-butanedione (**2**) and acetoin (**10**). Of particular importance may be the similarities observed among the neutral/basic aroma components. All three wines were equally impacted by esters (**1**, **3**, **5**, and **8**, *fruity*), 2/3-methyl-1-butanol (**7**, *malty, sour*), β-damascenone (**17**, *cooked apple*) and 2-phenylethanol (**19**, *rosy*). Based on their common occurrence these compounds appear to be necessary and characteristic components of Chardonel wine. In addition, the acidic aroma components, in particular compounds **26** and **29**, also may contribute to Chardonel wine flavor.

AEDA of CL3 confirmed the predominance ($\log_3$FD factors ≥ 3) of compounds **3**, **7**, **19**, **26** and **29** and indicated the potential importance of methional (**12**, cooked potato), phenylacetic acid (**31**, *rosy/plastic*) and 2/3-methyl butanoic acid (**24**, *dried fruit/sweaty*) in the overall aroma profile of Chardonel wine (Table III).

H-SPME-GCO

The LLCE-GCO technique discussed in the previous section allowed for the analysis of aroma constituents of intermediate and low volatility, but highly volatile compounds may be fully or partially lost during workup of the aroma extract or due to coelution with the solvent peak during analysis. On the other hand, a headspace-based technique like H-SPME-GCO is capable of detecting compounds of high, intermediate and low volatility, but compound polarity may affect sampling efficiency; e.g. H-SPME did not effectively extract the very polar-low volatility constituents **23-32**. Therefore, with respect to sampling efficiency neither technique has a clear advantage over the other. However, H-SPME is fast becoming the method of choice for wine volatiles analysis due to its high sensitivity, speed and ease of use (especially in regard to sample handing considerations). Furthermore, with rigorous calibration using an appropriate sample mimic matrix H-SPME can provide very accurate quantification.

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Table II. Comparison of Aroma Components of Wines CL1, CL2 and CL3 by Gas chromatography-Olfactometry and FDT

			FDT[b]		
No.	*Compound*	*Odor Description*[a]	*CL1*	*CL2*	*CL3*
	Neutral/basic fraction				
1	Ethyl 2-methylpropanoate	*fruity*	2	2	2
2	2,3-Butanedione	*2,3-butanedione*	- -[c]	1	- -
3	Ethyl butanoate	*fruity, bubble gum*	3	3	3
5	Ethyl 3-methylbutanoate	*fruity, blueberry*	3	3	4
6	3-Methyl-1-butyl acetate	*fruity, banana*	- -	2	- -
7	2/3-Methyl-1-butanol	*malty, sour*	4	4	4
8	Ethyl hexoate	*fruity, apple*	3	2	2
10	Acetoin	*buttery, sour*	2	3	- -
12	Methional	*potato*	3	2	3
13	Unknown (RI=1546)[d]	*yeasty*	4	1	1
16	Methionol	*cooked potato*	4	- -	2
17	β-Damascenone	*cooked apple*	4	4	4
18	Guaiacol	*smoky*	3	- -	- -
19	2-Phenylethanol	*rosy*	4	4	4
20	4-Ethylphenol	*phenolic*	4	- -	- -
22	4-Vinyl guaiacol	*spicy, cloves*	2	- -	- -
	Acidic fraction				
23	Butanoic acid	*cheesy, fecal*	1	2	2
24	2/3-Methylbutanoic acid	*dried fruit, sweaty*	2	2	2
26	DMHF[e]	*burnt sugar*	3	4	3
27	EMHF[f]	*burnt sugar*	1	3	3
29	Sotolon[g]	*spicy, curry*	4	3	3
31	Phenylacetic acid	*rosy, plastic*	3	1	1
32	Vanillin	*vanilla*	2	4	2

[a]Odor description perceived during GCO. [b]FDT, frequency of detection based on a panel of four judges. [c]Odor not detected. [d]Unidentified compound, retention index on DB-FFAP given in parentheses. [e]2,5-Dimethyl-4-hydroxy-3(*2H*)-furanone. [f]5-Ethyl-2-methyl-4-hydroxy-3(*2H*)-furanone. [g]2,5-Dimethyl-3-hydroxy-2(*5H*)-furanone.

Table III. Potent Odorants in Wine CL3 by Gas Chromatography-Olfactometry and Aroma Extract Dilution Analysis

No.	*Compound*	*Odor Property*[a]	*Average Log_3FD-Factor*[b]
	Neutral/basic fraction		
1	Ethyl 2-methylpropanoate	*fruity*	1.5
3	Ethyl butanoate	*fruity, bubble gum*	3.5
5	Ethyl 3-methylbutanoate	*fruity, blueberry*	2
6	3-Methyl-1-butyl acetate	*fruity, banana*	<1
7	2/3-Methyl-1-butanol	*malty, sour*	3
8	Ethyl hexoate	*fruity, apple*	2
12	Methional	*cooked potato*	3.5
17	β-Damascenone	*cooked apple*	3.5
19	2-Phenylethanol	*rosy*	3.5
	Acidic fraction		
23	Butanoic acid	*cheesy, fecal*	1
24	2/3-Methylbutanoic acid	*dried fruit, sweaty*	3
25	Hexanoic acid	*waxy, sweaty*	2
26	DMHF[c]	*burnt sugar*	4
27	EMHF[d]	*burnt sugar*	2
28	Octanoic acid	*waxy, sweaty*	2.5
29	Sotolon	*spicy, curry*	3
30	Decanoic acid	*waxy*	1
31	Phenylacetic acid	*rosy, plastic*	3.5
32	Vanillin	*vanilla*	1.5

[a] Odor description perceived during GCO. [b] Average log_3FD-factor (n = 3). [c] 2,5-Dimethyl-4-hydroxy-3(*2H*)-furanone. [d] 5-Ethyl-2-methyl-4-hydroxy-3(*2H*)-furanone. [e] 2,5-Dimethyl-3-hydroxy-2(*5H*)-furanone.

In initial experiments, we compared two types of SPME fibers (DVB/Carboxen™/PDMS vs. polyacrylate) for the analysis of wine CL3 by H-SPME-GCO (Table IV). Our data suggest that the fibers were nearly identical in their ability to isolate most of the aroma-active components of wine CL3, but the DVB/ Carboxen™/PDMS fiber yielded slightly higher aroma intensity scores for **12** and **14**, while the polyacrylate fiber give a higher score for **7**. The DVB/ Carboxen™/PDMS fiber was used for further analyses due to its higher recovery of highly volatile esters during GC-MS analysis (data not shown).

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Table IV. SPME Fiber Comparison for Analysis of Aroma Components of Chardonel Wine CL3

No.	Compound	Odor Property[a]	Average Odor Intensity[b] DVB/CAR/PDMS	Average Odor Intensity[b] Polyacrylate
1	Ethyl 2-ethylpropanoate	*fruity*	2.7	3
3	Ethyl butanoate	*fruity, bubble gum*	2	3
4	Ethyl 2-methylbutanoate	*fruity, berry*	2	2.3
5	Ethyl 3-methylbutanoate	*fruity, blueberry*	2.3	3
6	3-Methyl-1-butyl acetate	*fruity, banana*	2	2
7	2/3-Methyl-1-butanol	*malty, sour*	2.3	5
8	Ethyl hexanoate	*fruity, apple*	5.3	4.7
9	Hexyl acetate	*Fruit, apple*	0.3	0.3
11	Ethyl octanoate	*Fruity*	0.3	0.3
12	Methional	*cooked potato*	2.7	0.7
14	Ethyl sorbate	*fruity*	4	2.3
16	Methionol	*cooked potato*	0.3	- -[c]
17	β-Damascenone	*cooked apple*	4.3	4
19	2-Phenylethanol	*rosy*	4	4.3

[a]Odor description perceived during GCO. [b]Average odor intensity rated by GCO (n=3). [c]No odor detected.

Results of comparative H-SPME-GCO analysis of the four wines are shown in Table V. A total of 19 aroma-active compounds were detected. Except for linalool (**15**) and several esters (**4**, **9**, **11**, **14**), all of these compounds were detected by the aforementioned LLCE-GCO techniques. Linalool (**15**, *floral, honeysuckle*) was detected at low odor intensities in all wines except CL3. Linalool is generally not considered an important aroma component of non-muscat wines such as Chardonel and Chardonnay (*26*). A total of nine esters were detected. Among these, esters **9**, **11** and **14** were detected in CL3 only. The presence of a high level of ethyl sorbate (**14**) in CL3 suggests that an appreciable amount of sorbic acid was added to this wine (*27*). Aside from the above differences, the LLCE-GCO and H-SPME-GCO techniques gave comparable results.

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Table V. Potent Odorants in Wines CL1, CL2, CL3 and CY1 Detected by Static Headspace-Solid Phase Microextraction-Gas Chromatography-Olfactometry

No.	*Compound*	*Odor Property*[a]	*Average Odor Intensity*[b]			
			CL1	*CL2*	*CL3*	*CY1*
1	Ethyl 2-ethyl-propanoate	*fruity*	2.7	2.7	2.7	2
3	Ethyl butanoate	*fruity, bubble gum*	1.7	2.3	2	1.3
4	Ethyl 2-methyl-butanoate	*fruity, berry*	2.7	0.7	2	0.7
5	Ethyl 3-methyl-butanoate	*fruity, blueberry*	2.7	2	2.3	2.3
6	3-Methyl-1-butyl acetate	*fruity, banana*	1.7	2.7	2	2.7
7	2/3-Methyl-1-butanol	*malty, sour*	3.3	3	2.3	3
8	Ethyl hexanoate	*fruity, apple*	3.7	3.7	5.3	4
9	Hexyl acetate	*Fruit, apple*	- -[c]	- -	0.3	- -
11	Ethyl octanoate	*Fruity*	- -	- -	0.3	- -
12	Methional	*cooked potato*	0.3	0.7	2.7	0.7
14	Ethyl sorbate	*fruity*	- -	- -	4	- -
15	Linalool	*floral, honey*	1.3	1.7	- -	1
16	Methionol	*cooked potato*	- -	- -	0.3	0.3
17	β-Damascenone	*cooked apple*	4.7	3.3	4.3	4
18	Guaiacol	*smoky*	1.7	- -	- -	0.3
19	2-Phenylethanol	*rosy*	3.7	3.3	4	3
20	4-Ethylphenol	*spicy, phenolic*	2.7	- -	- -	- -
21	Unknown (RI=2120)[d]	*fuity, candy*	- -	1	- -	- -
22	4-Vinyl guaiacol	*spicy, cloves*	- -	0.7	- -	- -

[a] Odor description perceived during GCO. [b] Average odor intensity rated by GCO (n=3). [c] No odor detected. [d] Unidentified compound, retention index on DB-FFAP column in parenthesis.

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Table VI. Concentrations and Odor Activity Values of Selected Aroma Components of Wine Samples

No.	Compound	*Odor Threshold*[a]	*Concentration (ug/L)*[b]				*Odor Activity Value*[c]			
			CL1	*CL2*	*CL3*	*CY1*	*CL1*	*CL2*	*CL3*	*CY1*
	Esters									
1	Ethyl 2-ethylpropanoate	15 (*28*)	272	95.1	129	89.3	18	6	9	6
3	Ethyl butanoate	20 (*28*)	422	368	147	370	21	18	7	19
4	Ethyl 2-methylbutanoate	1 (*28*)	27.2	8.2	15.1	3.5	27	8	15	4
5	Ethyl 3-methyl-butanoate	3 (*28*)	25.1	9.7	18.9	- -[d]	8	3	6	- -
6	3-Methyl-1-butyl acetate	30 (*28*)	639	1770	436	1570	21	59	15	52
8	Ethyl hexanoate	5 (*28*)	775	999	721	785	155	200	144	157
11	Ethyl octanoate	2 (*28*)	2220	2230	1490	1350	148	149	99	90
14	Ethyl sorbate	300 (*27*)	- -	- -	574	- -	- -	- -	2	- -
33	Ethyl decanoate	200 (*29*)	1200	1491	898	612	6	7	4	3
	Alcohols and Ketones									
7	3-methyl-1-butanol	30000 (*28*)	37500	164000	170000	144000	1	5	6	5
16	Methionol (m/z=106)[e]	500 (*28*)	162	- -	616	439	<1	<1	1	1
19	2-phenylethanol	10000 (*28*)	21200	8940	23400	24400	2	1	2	2
17	β-Damascenone (m/z=69)	0.05 (*28*)	1.8	1.4	3.2	2.4	36	29	64	48
	Phenolics									
18	Guaiacol (m/z = 109)	10 (*28*)	18.5	- -	- -	11.8	2	- -	- -	1
20	4-Ethylphenol (m/z=107)	1000 (*30*)	1160	1.5	- -	5.9	1	<1	- -	- -
22	4-Vinylguaiacol	40 (*28*)	8.9	38.8	11.6	27.2	<1	<1	<1	<1
	Acids									
23	Butanoic acid (m/z = 60)	10000 (*28*)	205	158	157	139	<1	<1	<1	<1
24	3-Methylbutanoic acid (m/z=60)	3000 (*28*)	268	136	169	126	<1	<1	<1	<1

[a] μg/L in 10% w/w aqueous ethanol, reference in parentheses. [b] Average concentration (n = 2). [c] Odor activity value = concentration divided by odor threshold. [d] Not detected. [e] Number in parentheses = mass ion used in quantitation. [f] Not available.

Quantification Results

Concentrations and odor activity values (OAVs) for selected volatile constituents of the three Chardonel wines are given in Table VI. A total of 18 compounds were quantified by H-SPME-GC-MS. Aside from ethanol, 3-methyl-1-butanol (**7**) was the most abundant volatile component followed by 2-phenylethanol (**19**) and several esters (e.g. **6**, **8**, **11** and **33**). The OAVs in Table VI were calculated on the basis of published odor detection thresholds determined in aqueous ethanol. Based on their high OAVs, the most intense odorants in Chardonel wines consisted of eight esters (**1**, **3**, **4-6**, **8**, **11** and **33**) and β-damascenone (**17**), followed by 3-methyl-1-butanol (**7**) and 2-phenylethanol (**19**). Some very polar acidic components such as **25-32** were not quantified and their contribution based on their OAVs cannot be measured.

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Conclusions

This study revealed the predominant characteristic aroma components of Chardonel wine. LLCE-GCO (AEDA and FDT) allowed for the determination of intermediate and low volatilility aroma components, whereas H-SPME-GCO was capable of detecting components of high, intermediate and low volatility but was insensitive to very polar constituents (e.g. **25-32**). Both methods gave comparable results with respect to the potent aroma components of Chardonel wine. Due to its high sensitivity (especially for esters), ease of use, and high sample throughput capability, H-SPME-GC should be considered the method of choice for routine determination of Chardonel wine aroma composition

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Chapter 28

Antioxidant Effects of Tetrahydro-β-carboline Derivatives Identified in Aged Garlic Extract

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1,2,3,4-Tetrahydro-β-carboline derivatives (THβCs) are formed through Pictet-Spengler chemical condensation between tryptophan and aldehydes during food production, storage and processing. In the present study, in order to identify the antioxidants in aged garlic extract (AGE), we fractionated it and identified four THβCs; 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (MTCC) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (MTCdiC) in both diastereoisomers using liquid chromatography-mass spectrometry (LC-MS). Interestingly, these compounds were not detected in raw garlic, but the contents increased during the natural aging process of garlic. In *in vitro* assay systems, all of these compounds have shown strong hydrogen peroxide scavenging activities. (1S,3S)-MTCdiC was found to be stronger than the common antioxidant, ascorbic acid. MTCC and MTCdiC inhibited AAPH-induced lipid peroxidation. Both MTCdiCs also inhibited lipopolysaccharide (LPS)-induced nitrite production from murine macrophages at 10-100 μM. Thus, these compounds are potent antioxidants in AGE, and hence may be useful for prevention of disorders associated with oxidative stress.

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Introduction

1,2,3,4-Tetrahydro-β-carboline derivatives (THβCs) are formed by Pictet-Spengler condensation of tryptophan with aldehydes or α-oxo acids (*1*). These compounds are naturally occurring substances formed during food production, storage and processing. THβCs have been identified in soy sauces, beers, wines, chocolate and cocoa (*2-5*). Biological activities of these compounds have been demonstrated to show antioxidant property (*6,7*), to inhibit platelet aggregation (*2*) and monoamine oxidase, to alter the re-uptake of biogenic amine, and to interfere with benzodiazepine receptor (*8,9*).

Garlic (*Allium sativum* L.) has been considered a valuable healing agent by people of many different cultures for thousands of years. Even today, it is commonly used for its medicinal benefit throughout the world, especially Eastern Europe and Asia. It has been considered that the medicinal and beneficial properties may be attributed to specific constituents found in garlic and its extracts, and many studies suggest that organosulfur compounds are responsible for the biological activities (*10*). Among the many commercial garlic products, aged garlic extract (AGE) is uniquely manufactured by a more than 10-months natural aging process. The extract contains unique and bioactive organosulfur compounds such as *S*-allylcysteine (SAC) and *S*-allylmercaptocysteine (SAMC). It has previously been reported that AGE and its major organosulfur compounds, SAC and SAMC, show a variety of biological activities including antioxidant (*11-13*), cancer-preventing (*14*), anti-atherogenic (*15*) and anti-platelet aggregation (*16, 17*) properties. In the present study, in order to identify the antioxidants, AGE was fractionated using the hydrogen peroxide scavenging assay. The four alkaloids, THβCs; 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids (MTCC; **1a/b**) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (MTCdiC; **2a/b**) in both diastereoisomers were found by liquid chromatography-mass spectrometry (LC-MS) analysis and their fragmentation experiments (MS/MS) using an ion trap mass spectrometer. We further determined the antioxidant effects of these compounds using *in vitro* assay systems, and also analyzed the changes in concentrations of the alkaloids during the natural aging process of garlic using LC-MS. We now report that all of the MTCCs and MTCdiCs identified in AGE show potent hydrogen peroxide scavenging properties. Further, (1S, 3S)-MTCdiC is a more powerful hydrogen peroxide scavenger than the common antioxidant, ascorbic acid. We also show that these compounds inhibit 2,2'azobis (2-amidinopropane) hydrochloride (AAPH)-induced lipid peroixdation, and MTCdiCs inhibits LPS-induced nitrite production from macrophages. These data indicate that not only organosulfur compounds but

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also these alkaloids formed during the natural aging process may contribute to the antioxidant effects of AGE.

Materials and Methods

Aged garlic extract (AGE). AGE was manufactured under a license issued by the Ministry of Health and Welfare of Japan, and formulated as follows; sliced raw garlic (*Allium sativum* L.) was dipped into the aqueous ethanol, and extracted for more than 10 months at room temperature (*11*). AGE used for these experiments contained *S*-allylcysteine (SAC) in the range of 1.6-2.4 mg/g (calculated as dry weight)(*18*).

Chemicals. Linoleic acid, hydrogen peroxide, 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH), sodium dodecyl sulfate (SDS), lipopolysaccharide (LPS), acetonitrile, hydrochloric acid (HCl), sodium chloride (NaCl), sodium hydroxide (NaOH), magnesium sulfate ($MgSO_4$), sulfuric acid, pyruvic acid, *p*-toluene sulfonic acid monohydrate, sodium hydrogen carbonate, ammonium hydroxide (NH_4OH), chloroform ($CHCl_3$), dimethyl sulfoxide (DMSO) and trifluoroacetic acid (TFA) were purchased from Wako Pure Co. (Osaka, Japan). Methanol (MeOH), ethanol (EtOH), dichloromethane (CH_2Cl_2), diethyl ether (Et_2O), acetaldehyde were obtained from Yoneyama Yakuhin Kogyo Co. (Osaka, Japan). L-Tryptophan was from Peptide Institute Inc. (Osaka, Japan). Ethyl pyruvate was obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 2,2'-Azino-di-[3-ethyl-benzthiazoline-6-sulfonic acid] (ABTS) and horseradish peroxidase were purchased from Boehringer Mannheim Co. (Indianapolis, IN). Griess Reagent System Kit was obtained from Promega Co. (Madison, WI). Dulbecco's modification of Eagle's medium (DMEM) and foetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO). Penicillin-streptomycin solution was from Life Technologies (Rockville, MD). Oxygen electrode was supplied by Able Co. (Tokyo, Japan).

Cell Lines. Murine macrophage cell line (J774.A) was obtained from Japan Health Sciences Foundation (Tokyo, Japan). J774.A was grown in DMEM with 10% FBS. The media was supplemented with 200 U/mL penicillin and 0.2 mg/mL streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3-4 days before experimental use. Viability of cells used throughout the experiments was always greater than 95% as determined by trypan blue exclusion.

AGE Fractionation. AGE fractionation was performed according to the method of Ryu *et al.* (*19*) and summarized in Figure 1. AGE was partitioned

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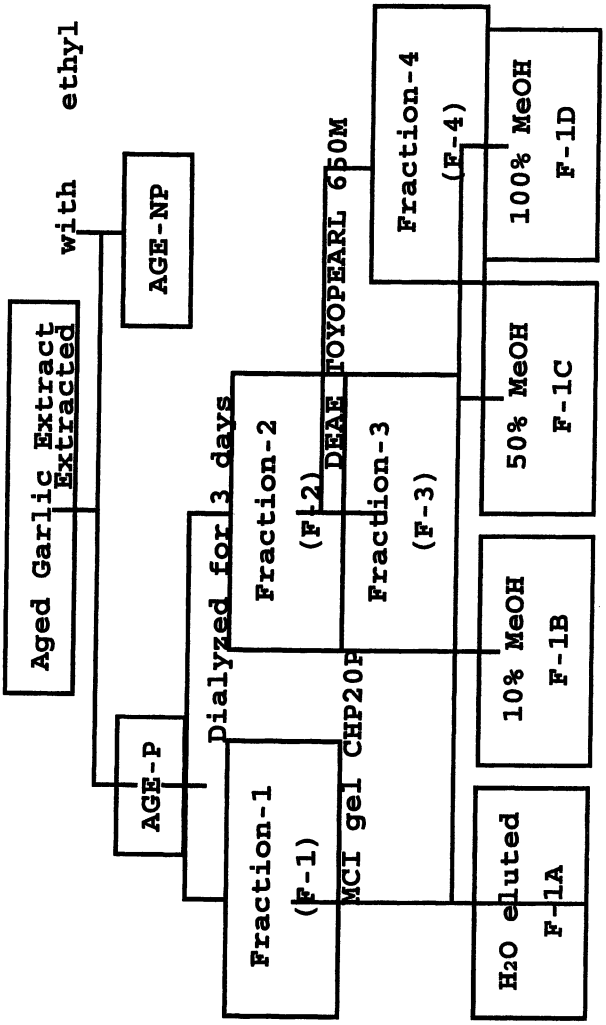
Aged Garlic Extract
Extracted with ethyl
AGE-NP
AGE-P
Dialyzed for 3 days
Fraction-1
(F-1)
Fraction-2
(F-2)
MCI gel CHP20P
DEAE TOYOPEARL 650M
Fraction-3
(F-3)
Fraction-4
(F-4)
H₂O eluted
F-1A
10% MeOH
F-1B
50% MeOH
F-1C
100% MeOH
F-1D

Figure 1. *Procedure for aged garlic extract fractionation*

between ethyl acetate and water. The water layer (AGE-P) was dialyzed against distilled water using Spectra/Por membrane with a molecular weight cut off (MWCO) of 1,000 Da (Spectrum, Houston, TX) at 4°C for 3 days to give fraction-1 [F-1; MW: <1000 Da] and fraction-2 [F-2; MW: >1000 Da]. F-2 was chromatographed on DEAE TOYOPEARL 650M (Tosoh, Tokyo, Japan) with 0.05 M Tris-HCl buffer (pH 8.0) and 0.05 M Tris-HCl buffer (pH 8.0) containing 2M NaCl to give fraction-3 (F-3; sugars) and fraction-4 (F-4; proteins). F-1 was subjected to reversed-phase column chromatography on MCI gel CHP 20P (Mitsubishi Chemical, Tokyo, Japan) with aqueous MeOH in stepwise gradient mode (water, 10% MeOH, 50% MeOH to MeOH) to give 4 fractions F-1A to F-1D (Figure 1). F1-D from MeOH elute was further fractionated on silica gel eluting with $CHCl_3$ followed by gradient mixtures of $CHCl_3$-MeOH and MeOH, and given subfractions were chromatographed on reversed-phase HPLC using MeOH/water (1:9, v/v) as eluent.

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High Performance Liquid Chromatography (HPLC). In order to obtain subfractions, AGE fractionation was performed using SCL-6B HPLC system with SPD-6A UV spectrometric detector and LC-6A pump (Shimadzu, Kyoto, Japan). A 300 mm x 21.5 mm, 10 μm, TSK gel ODS-80TM (Tosoh, Tokyo, Japan) was used for HPLC separation. Flow rate was 9 mL/min, UV detection was carried out at 210 nm, and MeOH/water (1:9, v/v) was used as eluent.

Mass Spectrometric Analysis of THβCs. Chromatographic separation for LC-MS was performed on a Capcell Pak C18UG120 (75 mm x 2.0 mm i.d., 3 μm) (Shiseido, Tokyo, Japan). Gradient elution was performed using HP-1100 binary pump (Hewlett-Packard, Palo Alto, CA). Solvent A was 0.05% trifluoroacetic acid (TFA) in water, solvent B was 0.05% TFA in water/acetonitrile (1:1, v/v) and the linear gradient was programmed as follows; t=0 min 20% solvent B, t=20 min 60% solvent B, t=30 min 100% solvent B. The flow rate was 0.2 mL/min and injection volume was 5 μL, respectively. The mass spectrometric detector was an ion trap mass spectrometer LCQ (Thermoquest, Palo Alto, CA) equipped with atmospheric pressure chemical ionization (APCI). An optimal condition of the APCI source parameter was obtained at the following values: sheath gas flow rate was 60 (arbitrary unit defined by the software), vaporizer temperature was 450°C, discharge current was 5 μA, capillary voltage was 3 V, capillary temperature was 150°C, scanning mode was positive and scan range was 100-300 amu. Analytical data were acquired using *Xcalibur* software (version 1.0 SR1). Fragmentation experiments (MS/MS) was performed in the trap with helium as the collision gas and the relative collision energy was set at 35%.

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Nuclear Magnetic Resonance (NMR) Spectroscopy. ^{1}H- and ^{13}C-NMR and NOE experiments were performed on a JEOL JNM-ECP500 (JEOL, Tokyo, Japan) apparatus at 500 MHz and 125 MHz, respectively.

Synthesis of Reference Compounds. Synthesis of THβCs was achieved according to the methods described elsewhere (*4, 20*).

(1R, 3S)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1a) and (1S, 3S)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1b). L-Tryptophan (10 g, 48.9 mmol) was dissolved in 100 mL of water. After addition of 1 mL of sulfuric acid and acetaldehyde (4 mL, 71.5 mmol), the reaction mixture was stirred at room temperature for 12 h. The pH was adjusted to 5 with 1N NaOH. The precipitate was collected by filtration, washed with water and EtOH, and dried to yield 8.1 g of 1b. The filtrate was concentrated to 20 mL and an additional 0.9 g of 1b (total yield of 80%). The filtrate was concentrated to 10 mL and cooled to 6°C for 12 h. The white crystals were collected by filtration, washed, and dried to yield 0.45 g (4%) of 1a. Assignment of NMR signals was confirmed by GROESY, H-H COSY, HMQC and HMBC experiments. LC-MS, [M+H]$^{+}$ m/z 231; MS/MS, m/z 214, 188, 158. 1a: ^{1}H-NMR 500 MHz, DMSO-d6 δ 1.57 (d, J=6.9 Hz, 3H, Me), 2.95 (dd, J=7.8, 15.6 Hz, 1H, H-4), 3.08 (dd, J=5.5, 16.0 Hz, 1H, H-4), 3.79 (dd, J=5.5, 7.8 Hz, 1H, H-3), 4.63 (dd, J=6.9, 13.8 Hz, 1H, H-1), 6.98 (dd, J=7.3, 7.8 Hz, 1H, H-6), 7.07 (dd, J=6.9, 8.3 Hz, 1H, H-7), 7.32 (d, J=8.3 Hz, 1H, H-8), 7.43 (d, J=7.8 Hz, 1H, H-5), 11.05 (s, 1H, H-9); ^{13}C-NMR (125 MHz, DMSO-d6) δ18.9 (Me), 23.3 (C-4), 47.0 (C-1), 53.4 (C-3), 106.1 (C-4a), 111.7 (C-8), 118.5 (C-5), 119.2 (C-6), 121.8 (C-7), 126.7 (C-4b), 133.2 (C-9a), 136.7 (C-8a), 170.2 (COOH). 1b: ^{1}H-NMR 500 MHz, DMSO-d6 δ 1.64 (d, J=6.9 Hz, 3H, Me), 2.86 (dd, J=13.8, 14.2 Hz, 1H, H-4), 3.23 (dd, J=4.6, 16.0 Hz, 1H, H-4), 3.92 (dd, J=4.6, 11.5 Hz, 1H, H-3), 4.60 (dd, J=7.8, 14.2 Hz, 1H, H-1), 7.01 (dd, J=7.3, 7.8 Hz, 1H, H-6), 7.11 (dd, J=7.3, 7.8 Hz, 1H, H-7), 7.36 (d, J=7.8 Hz, 1H, H-8), 7.46 (d, J=7.8 Hz, 1H, H-5), 11.16 (s, 1H, H-9); ^{13}C-NMR (125 MHz, DMSO-d6) δ 17.3 (Me), 23.5 (C-4), 49.8 (C-1), 57.3 (C-3), 106.6 (C-4a), 111.9 (C-8), 118.6 (C-5), 119.5 (C-6), 122.1 (C-7), 126.5 (C-4b), 132.3 (C-9a), 137.0 (C-8a), 170.6 (COOH).

(1R, 3S)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (2a). L-Tryptophan (3 g, 14.7 mmol) was dissolved in 40 mL of water. After addition of 0.5 mL sulfuric acid and pyruvic acid (1.32 g, 15 mmol), the reaction mixture was stirred at room temperature for 12 h. The precipitate was collected by filtration, washed with water, EtOH and Et2O, and dried to yield 2.7 g (67%) of **2a**. Assignment of NMR signals was confirmed by GROESY, H-H COSY, HMQC and HMBC experiments. LC-MS, [M+H]$^{+}$ *m/z* 275; MS/MS, *m/z* 258,

231, 202, 159. ^{1}H-NMR 500 MHz, DMSO-*d6* δ 1.73 (s, 3H, Me), 2.87 (dd, *J*=11.5, 15.1 Hz, 1H, H-4), 3.13 (dd, *J*=4.6, 15.6 Hz, 1H, H-4), 4.11 (dd, *J*=4.6, 11.5 Hz, 1H, H-3), 6.96 (dd, *J*=7.3, 7.8 Hz, 1H, H-6), 7.05 (dd, *J*=7.3, 7.8 Hz, 1H, H-7), 7.40 (d, *J*=8.3 Hz, 1H, H-5 and H-8), 10.71 (s, 1H, H-9); ^{13}C-NMR (125 MHz, DMSO-*d6*) δ 23.6 (C-4), 24.2 (Me), 51.7 (C-3), 60.2 (C-1), 104.5 (C-4a), 111.7 (C-8), 117.7 (C-5), 118.6 (C-6), 121.2 (C-7), 125.7 (C-4b), 133.2 (C-9a), 136.2 (C-8a), 170.8 (COOH), 171.8 (COOH).

(1S, 3S)-1-Methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (2b). In order to prepare **2b**, diethyl ester of **2b** was synthesized as the first step. Ethyl pyruvate (2.4 g, 20.7 mmol), and *p*-toluene sulfonic acid monohydrate (6.6 g, 34.4 mmol) were added to the L-tryptophan ethyl ester (4 g, 17.2 mmol) dissolved in 100 mL of EtOH. The mixture was heated to reflux for 2 h and cooled to room temperature. The solvent was evaporated *in vacuo*. Given residue was dissolved in 100 mL of saturated sodium hydrogen carbonate solution and extracted with CH_2Cl_2, and the combined extracts were dried with $MgSO_4$. The diastereomer was separated by column chromatography (eluent: CH_2Cl_2/MeOH 50:1, v/v). After concentration of the first eluate *in vacuo*, the residue was precipitated with Et_2O to yield 1.2 g (21%) of diethyl ester as a white solid. Prepared diethyl ester (0.5 g, 1.51 mmol) was dissolved in 15 mL of EtOH to synthesize **2b**. After addition of 2 mL of NaOH (0.18 g, 4.52 mmol) solution, the reaction mixture was stirred at room temperature for 12 h. The solution was applied to Dowex 50W column (H^+ form). After the column was washed with water, the product was eluted with 2N NH_4OH. This eluate was concentrated *in vacuo*. The residue was precipitated with Et_2O to yield 0.3 g (72%) as a yellow powder. Assignment of NMR signals was confirmed by GROESY, H-H COSY, HMQC and HMBC experiments. LC-MS, $[M+H]^+$ *m/z* 275; MS/MS, *m/z* 258, 231, 202, 159. ^{1}H-NMR 500 MHz, DMSO-*d6* δ 1.77 (s, 3H, Me), 2.78 (dd, *J*=12.8, 15.1 Hz, 1H, H-4), 3.10 (dd, *J*=3.7, 15.6 Hz, 1H, H-4), 3.78 (dd, *J*=4.1, 11.9 Hz, 1H, H-3), 6.95 (dd, *J*=7.3, 7.8 Hz, 1H, H-6), 7.04 (dd, *J*=6.9, 7.3 Hz, 1H, H-7), 7.34 (d, *J*=7.8 Hz, 1H, H-8), 7.40 (d, *J*=7.8 Hz, 1H, H-5), 11.03 (s, 1H, H-9); ^{13}C-NMR (125 MHz, DMSO-*d6*) δ 22.9 (C-4), 24.4 (Me), 56.8 (C-3), 62.8 (C-1), 105.7 (C-4a), 111.8 (C-8), 118.3 (C-5), 118.9 (C-6), 121.4 (C-7), 126.1 (C-4b), 134.1 (C-9a), 137.2 (C-8a), 169.9 (COOH), 170.3 (COOH).

Hydrogen Peroxide Scavenging Assay. The scavenging effects of obtained fractions or THβCs identified in AGE on hydrogen peroxide were determined according to the method of Okamoto *et al.* with slight modification (*21*). Briefly, 10 μL of 500 μM hydrogen peroxide, 10 μL of sample or H_2O as a control, 40 μL of 150 U/mL horseradish peroxidase, and 40 μL of 0.1% ABTS were added to 120 μL of 0.1 N phosphate buffer (pH 6.0). The solution was

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then incubated at 37°C for 15 min. Absorbance at 414 nm was measured using Multiskan Ascent (Labsystems, Helsinki, Finland).

AAPH-induced Lipid Peroxidation. AAPH is a water-soluble and azo compound, which works as a radical initiator and causes lipid peroxidation. The effects of THβCs on AAPH-induced lipid peroxidation were determined according to the method described before (*22, 23*). Briefly, 1.4 mL of 50 mM linoleic acid-50 mM SDS micelles solution was added to the reaction vessel equipped with an oxygen electrode, kept at 37°C and stirred to saturate oxygen. Various concentrations of THβCs (0.1, and 1 mM) or H_2O as a control was added to the mixture, kept for 2 min, and 40 μL of 0.5 M AAPH as a radical initiator were added to the system. The amounts of oxygen consumed in the mixture were monitored for 20 min, and compared with control.

Determination of Nitrite Production from Macrophages. The effects of THβCs on nitrite production from macrophages were determined according to the methods as described before (*13, 24*). Harvested J774.A cells (2×10^5/well) were incubated for 2 h in 96-well plates. After incubation, the media were removed, and cells were washed and incubated with 10-250 μM of THβCs in DMEM, and 10 μg/mL of LPS for 20 h at 37°C. Nitrite was determined in supernatant using the Griess Reagent System Kit according to the manufacture's instruction. This assay is based on converting nitrite into a deep purple azo compound using Griess Reagent. The intensity of color is proportional to the concentration of nitrite. Absorbance was measured at 540 nm with a 96-well plate ELISA reader (Labsystems, Helsinki, Finland). Nitrite solution included in the assay kit was used as a standard.

Statistical Analysis. Data were analyzed using the one-tailed Student's t-test (Microsoft Excel), and results were expressed as the mean ± SE. A *p* value of less than 0.05 was considered significant.

Results

Table I shows the scavenging effects of AGE and its fractions on hydrogen peroxide. At 1 mg/mL, AGE and its fractions, AGE-P, F-1, F-2, F-3 and F-4, showed the scavenging activities, 75.2%, 58.8%, 65.5%, 54.7%, 0%, and 97.2%, respectively. From the obtained scavenging activities and weights of fractions, F-1 was further fractionated on MCI gel CHP 20P with aqueous MeOH in stepwise gradient mode to give F-1A to F-1D. At 0.1 mg/mL, F-1C and F-1D showed 100% scavenging of hydrogen peroxide. However, F-1, F-1A and F-1B did not show any scavenging activities. In two bioactive fractions, F-1D from

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MeOH eluent was further repeated column chromatography on silica gel and reversed-phase HPLC and given 13 subfractions. After these subfractions were analyzed using LC-MS and MS/MS, assumed compounds were synthesized, analyzed using LC-MS, MS/MS, ^{1}H- and ^{13}C-NMR, and these alkaloids were identified as bioactive compounds. Table II shows THβCs identified in AGE; MTCCs (**1a/b**) and MTCdiCs (**2a/b**) in both diastereoisomers. As a representative example, the profile of MTCCs (**1a/b**) and MTCdiCs (**2a/b**) from AGE analysis is outlined in Figure 2, and mass spectrum and product ion (MS/MS) spectrum of MTCCs (**1a/b**) and MTCdiCs (**2a/b**) were shown in Figures 3 and 4. The protonated molecular ions were obtained at *m/z* 231 for the diastereoisomers, MTCCs (**1a/b**), and at *m/z* 275 for MTCdiCs (**2a/b**). Product ion spectra of **1a/b** as obtained by MS/MS show *m/z* 158. Product ions *m/z* 158 were formed by neutral loss of the iminoacetic acid moiety $C_2H_3NO_2$ (-73 amu) due to retro-Diels-Alder fragmentation (*4*). Product ion spectra of **2a/b** as obtained by MS/MS show *m/z* 231, 202 and 159. Product ions *m/z* 231 apparently resulted from the loss of CO_2 (-44 amu) characteristic for carboxylic acid; product ions *m/z* 202 were formed by neutral loss of the iminoacetic acid moiety $C_2H_2NO_2$ (-73 amu) due to retro-Diels-Alder fragmentation; product ions *m/z* 159 were indicative of the loss of the indole moiety (*4*). Figure 5A and B show the changes in concentrations of **1a/b** and **2a/b** during the natural aging process. In raw garlic, all of these compounds were not detected since they were either not present or below the detection limit [(1R, 3S)-MTCC (**1a**): 74.5 ng/g; (1S, 3S)-MTCC (**1b**): 86.1 ng/g; (1R, 3S)-MTCdiC (**2a**): 1.67 μg/g; (1S, 3S)-MTCdiC (**2b**): 1.54 μg/g]. However, all of these compounds were formed from the beginning of the natural aging process, and remarkably increased between 4 and 10 months. MTCdiCs (**1a/b**) further increased in concentrations after 10 months of aging, while MTCCs (**2a/b**) plateaued at 10 months (Figure 5A).

Scavenging effects of MTCCs (**1a/b**) and MTCdiCs (**2a/b**) on hydrogen peroxide are shown in Table III. At 250 μM, (1R, 3S)- or (1S, 3S)-MTCC scavenges hydrogen peroxide 49.0% or 35.4%, respectively. However, scavenging activities of MTCdiCs (**2a/b**) are about 5 to 10 times stronger than MTCCs (**1a/b**). Especially, (1S, 3S)-MTCdiC (**2b**) has shown stronger activity than the common antioxidant, ascorbic acid. Table IV shows the effects of MTCCs and MTCdiCs on AAPH-induced lipid peroxidation. In this experiment, the strongest hydrogen peroxide scavenger, (1S, 3S)-MTCdiC (**2b**) and its mono-carboxylate, (1S, 3S)-MTCC (**1b**) were used. Incubation of linoleic acid-SDS micelle solution with AAPH for 20 min resulted in a significant O_2 consumption and caused lipid peroxidation. Co-incubation with (1S, 3S)-MTCC (**1b**) or (1S, 3S)-MTCdiC (**2b**) inhibited AAPH-induced lipid peroxidation 30.7% or 28.6% at 1 mM and 10.3% or 10.0% at 0.1 mM (Table IV). However, there was no difference between **1b** and **2b**, and the activities were not strong compared with the common antioxidant, butylated

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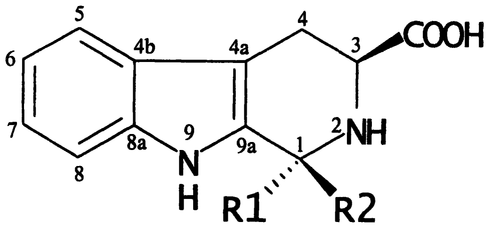
Table I. Scavenging effects of aged garlic extract and its fractions on hydrogen peroxide

Sample (g)	Weight Concentration		H_2O_2 (%)	Scavenging
	(mg/mL)	(nmol±SE)		(%)
Control	-		5.00	-
AGE	450	1	0.74±0.08	75.2
AGE-P	448	1	2.06±0.08	58.8
F-1	400	1	1.72±0.10	65.5
F-2	50	1	2.27±0.02	54.7
F-3	42	1	0	0
F-4	8	1	0.14±0.01	97.2
F-1	400	0.1	5.00	0
F-1A	380	0.1	5.00	0
F-1B	0.6	0.1	5.00	0
F-1C	1.5	0.1	0	100
F-1D	0.5	0.1	0	100

Data represent means ± SE of triplicate samples. H_2O_2: hydrogen peroxide, AGE: aged garlic extract, AGE-P: water-soluble fraction of AGE, F-1: MW <1000 fraction of AGE-P, F-2: MW>1000 fraction of AGE-P, F-3: sugar fraction in AGE, F-4: protein fraction in AGE, F-1A: H_2O elution of F-1, F-1B: 10% MeOH elution of F-1, F-1C: 50% MeOH elution of F-1, F-1C: 100% MeOH elution of F-1

Table II. 1,2,3,4-Tetrahydro-β-carboline derivatives identified in aged garlic extract



Compounds No.	R1	R2
(1R, 3S)-MTCC (**1a**)	CH_3	H
(1S, 3S)-MTCC (**1b**)	H	CH_3
(1R, 3S)-MTCdiC (**2a**)	CH_3	COOH
(1S, 3S)-MTCdiC (**2b**)	COOH	CH_3

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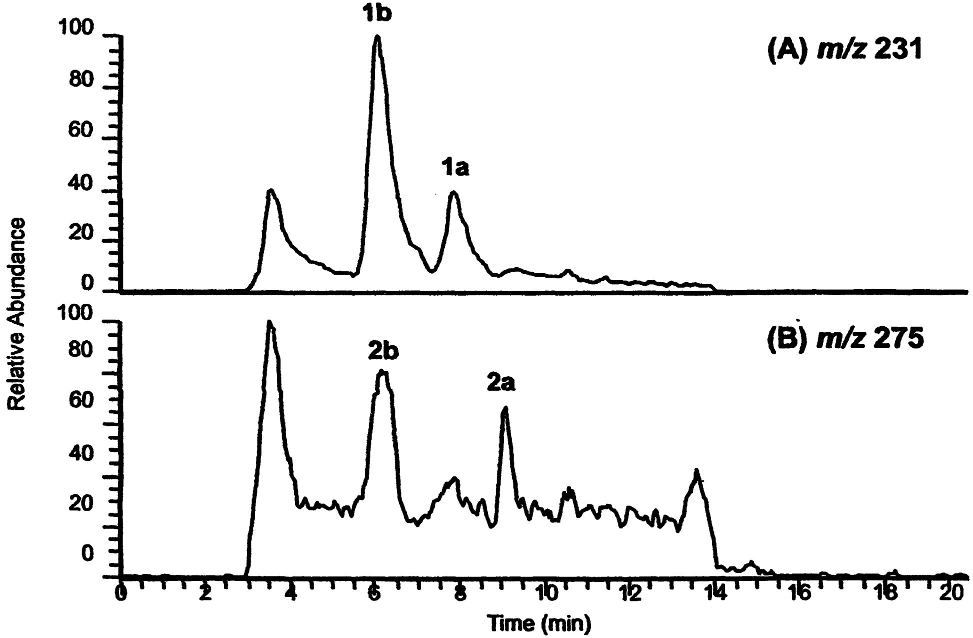

Figure 2. LC-MS ion chromatogram of m/z 231 and m/z 275 in aged garlic extract. Chromatographic condition was performed on Capcell Pak C18UG120 column (75 x 2.0 mm i.d., 3 μm) using stepwise gradient. Solvent A was 0.05% TFA in water, solvent B was 0.05% TFA in water/acetonitrile (1:1, v/v). HPLC condition was programmed as follows; t=0 min 20% solvent B, t=20 min 60% solvent B, t=30 min 100% solvent B. (A) mass chromatogram of m/z 231. (B) mass chromatogram of m/z 275.

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Table III. Scavenging Effects of Tetrahydro-β-carboline Derivatives Identified in Aged Garlic Extract on Hydrogen Peroxide

Sample	*Concentration (μM)*	*H_2O_2 (nmol±SE)*	*Scavenging (%)*
Control	-	5.00	-
(1R, 3S)-MTCC (**1a**)	250	2.55 ± 0.12	49.0
(1S, 3S)-MTCC (**1b**)	250	3.23 ± 0.04	35.4
(1R, 3S)-MTCdiC (**2a**)	50	3.28 ± 0.04	34.5
	100	2.38 ± 0.03	52.5
(1S, 3S)-MTCdiC (**2b**)	25	2.67 ± 0.01	46.6
	50	1.19 ± 0.04	76.2
Ascorbic acid	25	4.02 ± 0.07	19.7
	50	0.86 ± 0.04	82.7

Data represent means ± SE of triplicate samples. H_2O_2: hydrogen peroxide, MTCC: 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, MTCdiC: 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid

Table IV. Effects of Tetrahydro-β-carboline Derivatives Identified in Aged Garlic Extract on AAPH-induced Lipid Peroxidation

Sample	*Concentration (mM)*	*O_2 Consumption (μM ± SE)*	*Inhibition (%)*
Control	-	138.5±2.16	-
(1S, 3S)-MTCC (**1b**)	1	96.0±5.12*	30.7
	0.1	124.2±3.15*	10.3
(1S, 3S)-MTCdiC (**2b**)	1	98.9±5.40*	28.6
	0.1	124.6±1.35*	10.0
BHT	0.1	42.5	69.3

Data represent means ± SE of repeated studies (n=4). Significant difference compared with control without samples ($p<0.05$). BHT: butylated hydroxytoluene

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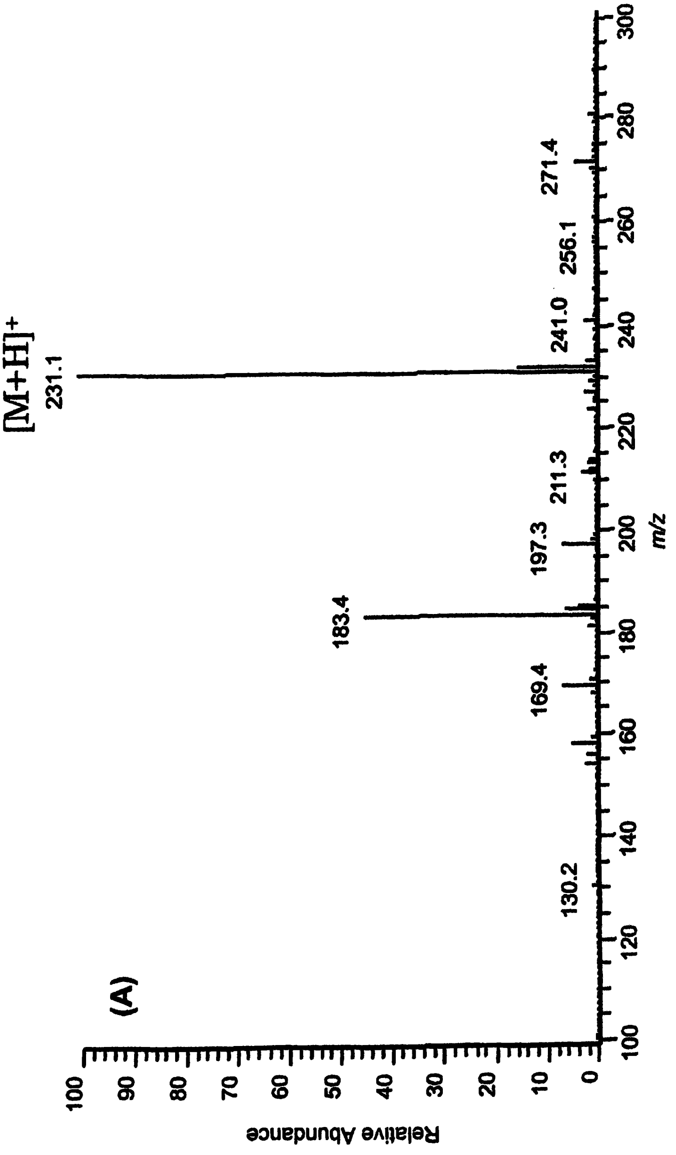

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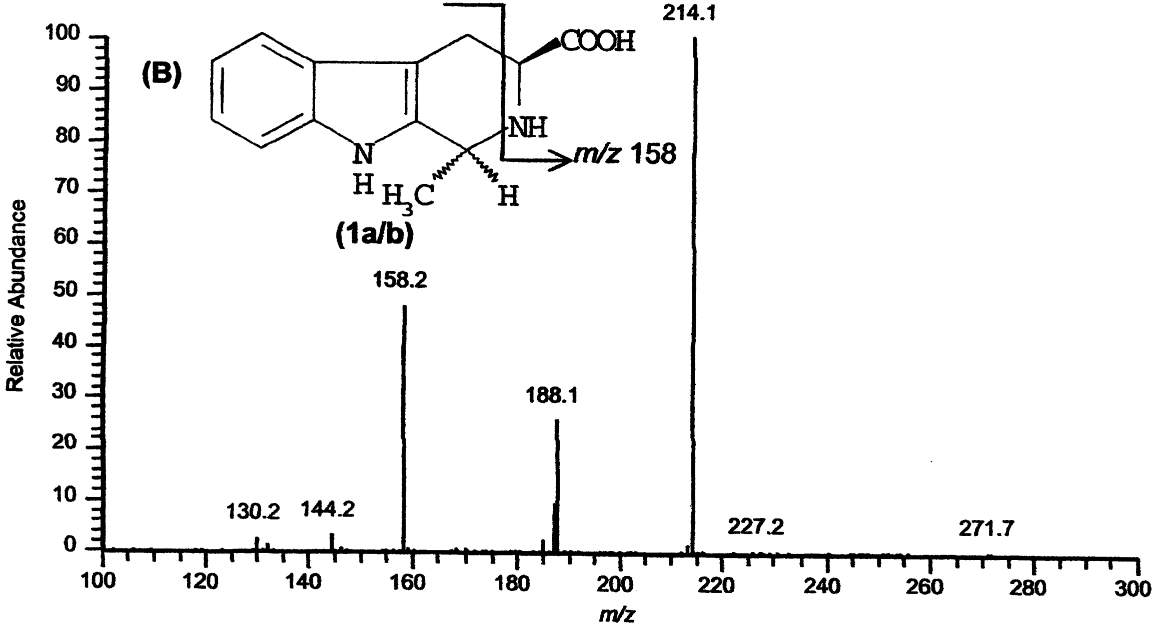

Figure 3. *Mass spectrum and product ion (MS/MS) spectra of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid,* ***1a/b****. (A) full scan mass spectrum. (B) product ion mass spectrum (precursor ion m/z 231 $[M+H]^+$).*

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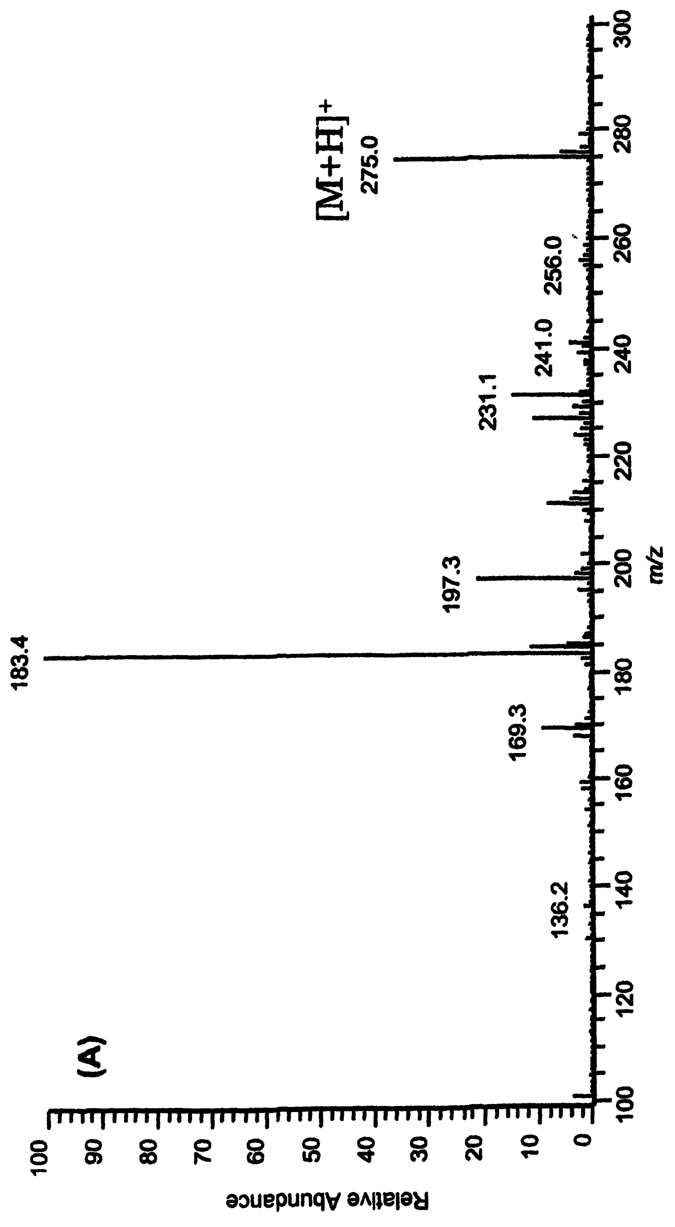

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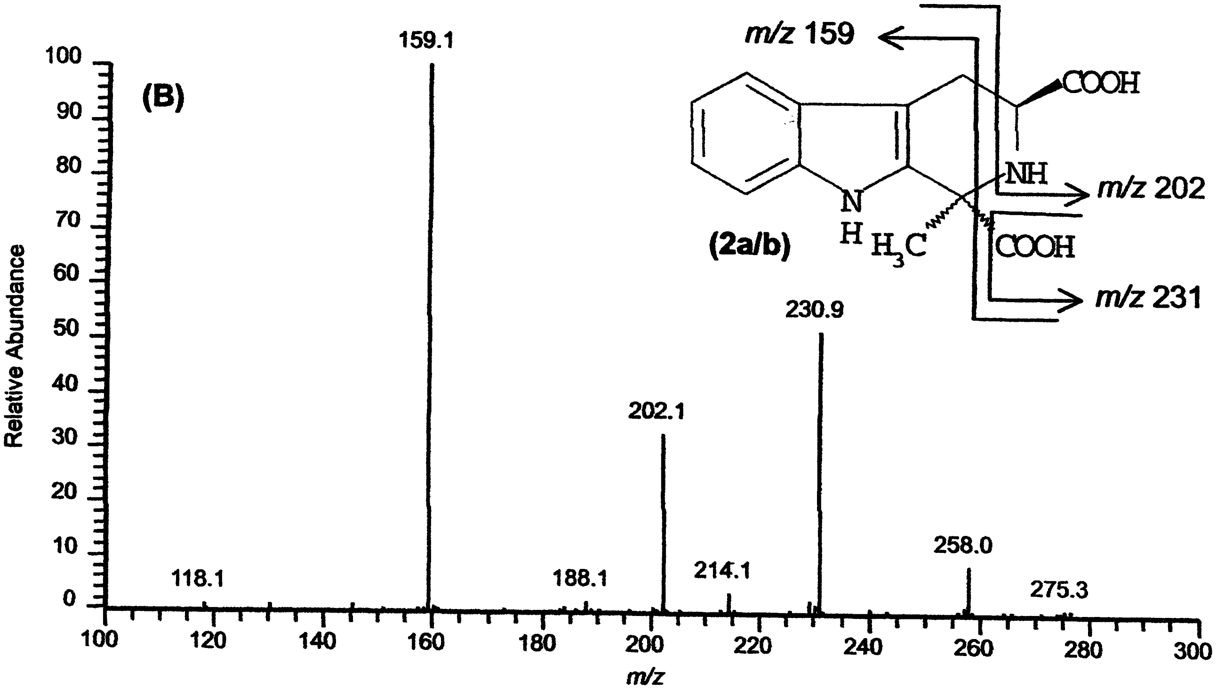

Figure 4. *Mass spectrum and product ion (MS/MS) spectra of 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid,* ***2a/b****. (A) full scan mass spectrum. (B) product ion mass spectrum (precursor ion m/z 275* $[M+H]^+$*).*

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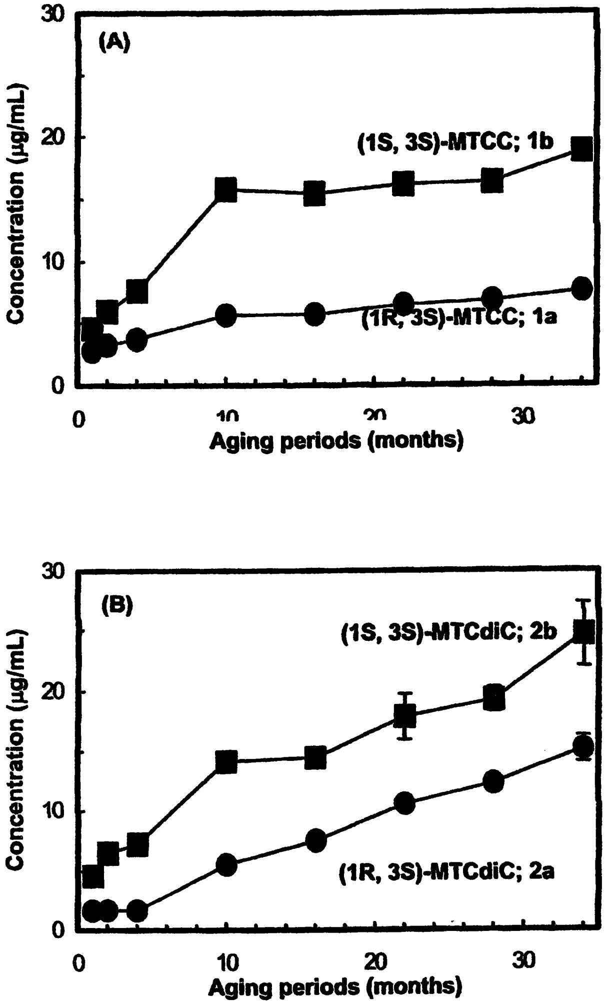

Figure 5. *Changes in concentrations of MTCCs (**1a/b**; A) and MTCdiCs (**2a/b**; B) during the natural aging process. Samples prepared during the natural aging process of garlic were analyzed by LC-MS. Data represent means ± SE of repeated studies (at least 3 times).*

hydroxytoluene (BHT). The effects of four MTCCs (**1a/b**) and MTCdiCs (**2a/b**) on LPS-induced nitrite production from macrophages are shown in Figure 6. Exposure of LPS (10 μg/mL) to J774 cells resulted in a significant release of nitrites. Co-incubation of J774 cells with (1R, 3S)-MTCdiC (**2a**) or (1S, 3S)-MTCdiC (**2b**) resulted in a significant inhibition of nitrite release from macrophages at 50 and 100 μM or 10 to 100 μM, respectively, though MTCCs (**1a/b**) didn't inhibit it at 250 μM.

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Discussion

Tetrahydro-β-carboline derivatives (THβCs) are formed through the Pictet-Spengler chemical condensation between tryptophan and aldehyde during food production, processing and storage. It has been previously reported that these compounds were identified and quantified in beer, wine, vinegar, sauce, soy sauce, fruit juice, chocolate and cocoa (*2-5*). Biological significance of THβCs has been reported on antioxidant effects (*6, 7*), anti-platelet aggregation (*2*), and neuromodulation such as inihibiting monoamine oxidase (MAO), biogenic amine (serotonin) uptake/release and benzodiazepine receptor binding (*8, 9*). In the present study, in order to identify antioxidants in aged garlic extract (AGE), which is a garlic product manufactured by a natural aging process for more than 10 months, we fractionated it, repeated chromatographies, and identified four THβCs in the extract; 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCC; **1a/b**) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (MTCdiC; **2a/b**) in both diastereoisomers. This is the first report on THβCs in processed garlic. In order to identify and quantify the compounds, LC-MS analyses were performed. LC-MS is a potent analytical method for the efficient analysis of minor compounds in complex materials. The excellent sensitivity and selectivity allowed specific detection of THβCs from coeluting compounds (*5*). Interestingly, all of these compounds were not detected in raw garlic due to less than detection limit. However, it has been shown THβCs are formed at the beginning of the natural aging process, remarkably increase between 4 and 10 months, and MTCdiCs (**2a/b**) further increase during aging, while the contents of MTCCs (**1a/b**) plateaued at 10 months.

It has been previously reported that the chemical formations of THβCs depend on storage time, pH, temperature and processing conditions. THβCs in fermented and maturated foodstuffs such as beer and wine are also believed to be related to the amount of aldehydes (*25-27*). Chemically, MTCCs (**1a/b**) and MTCdiCs (**2a/b**) are synthesized by the condensation between tryptophan and acetaldehyde or pyruvic acid, respectively. It is expected that acetaldehyde, which is a precursor to form **1a/b**, comes from alcohol as previously reported

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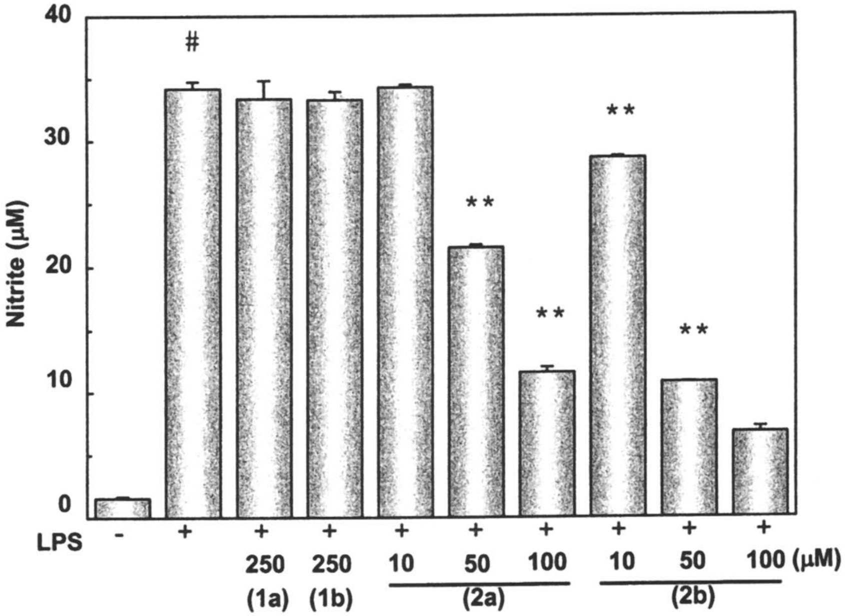

Figure 6. Effects of tetrahydro-β-carboline derivatives identified in aged garlic extract on LPS-induced nitrite production from murine macrophages. J744 cells were incubated with LPS (10 μg/mL) and various concentrations of MTCCs (1a/b) or MTCdiCs (2a/b) identified in AGE for 20h. Nitrite was measured using Griess Reagent System Kit. Data represent means ± SE of triplicate samples. Significant difference (p<0.01) compared with control without samples.

(*26, 27*). While, in the natural aging process of garlic, two pathways are expected to form pyruvic acid for determining **2a/b** contents in AGE; one is the alliin-allicin pathway, and another is via the Maillard reaction process. The former is the most common pathway in garlic. When raw garlic is cut or processed, a major organosulfur compound in raw garlic, alliin (*S*-allylcysteine sulfoxide), is converted into allicin (allyl 2-propenethiosulfinate) by a C-S lyase called alliinase, and pyruvic acid is formed as a by-product (*28, 29*). In the later process, 3-deoxyglucosone is a key compound, which is well-known as a Maillard reaction product. The compound has been reported to cause C3/C3 cleavage and form the pyruvaldehyde in a model Maillard reaction system (*30*). MTCdiCs (**2a/b**) in processed garlic may be formed by the non-enzymatic reaction between tryptophan and pyruvic acid and/or pyruvaldehyde formed in these two expected pathways (Figure 7).

Oxidative modification of lipids, proteins and DNA by reactive oxygen species plays an important role in a wide range of common diseases including cardiovascular diseases, inflammatory condition, and neurodegenerative diseases (*31-33*). Under oxidant-stressed condition, peroxides such as hydrogen peroxide and lipid peroxide change cell functions and interactions with surrounding cells. In the cardiovascular field, for instance, hydrogen peroxide damages endothelial cell membranes, reduces cell viability and induces lipid peroxidation (*34*). Also hydrogen peroxide serve as an important second messenger in the activation of the transcription factor NF-κB, which is associated with expression of cell adhesion factors, vascular cell adhesion molecules-1 (VCAM-1) and intercellular cell adhesion molecules-1 (ICAM-1) (*35, 36*). Lipid peroxidation in endothelial cells changes the permeability of cell membranes and ion efflux. Lysophosphatidylcholine (Lyso PC), which is a lipid peroxide composed of oxidized LDL, can be a trigger of inflammation that leads to the release of inflammatory mediators such as reactive oxygen species and cytokines (eg. TNF-α and IL-6). All of these mediators and oxidative stress cause endothelial cell injury and dysfunction, foam cells formation and smooth muscle cell proliferation, and eventually lead to the formation of atherosclerotic lesion. In the present study, in order to determine antioxidants in aged garlic extract (AGE), AGE was fractionated using hydrogen peroxide scavenging assay, and four alkaloids; MTCCs (**1a/b**) and MTCdiCs (**2a/b**) were identified. All of these compounds have shown the strong hydrogen peroxide scavenging activities. Especially, the activity of (1S, 3S)-MTCdiC (**2b**) was stronger than the common antioxidant, ascorbic acid. To elucidate the mechanism by which (1S, 3S)-MTCdiC (**2b**) scavenges hydrogen peroxide, we also analyzed the change of **2b** in the presence of hydrogen peroxide and peroxidase using LC-MS. The data showed **2b** was decarboxylated at 1 position and dehydrated between 1 and 2 position (data not shown), suggesting that this compound functions as an electron donor and scavenge hydrogen peroxide, and the presence of carboxyl

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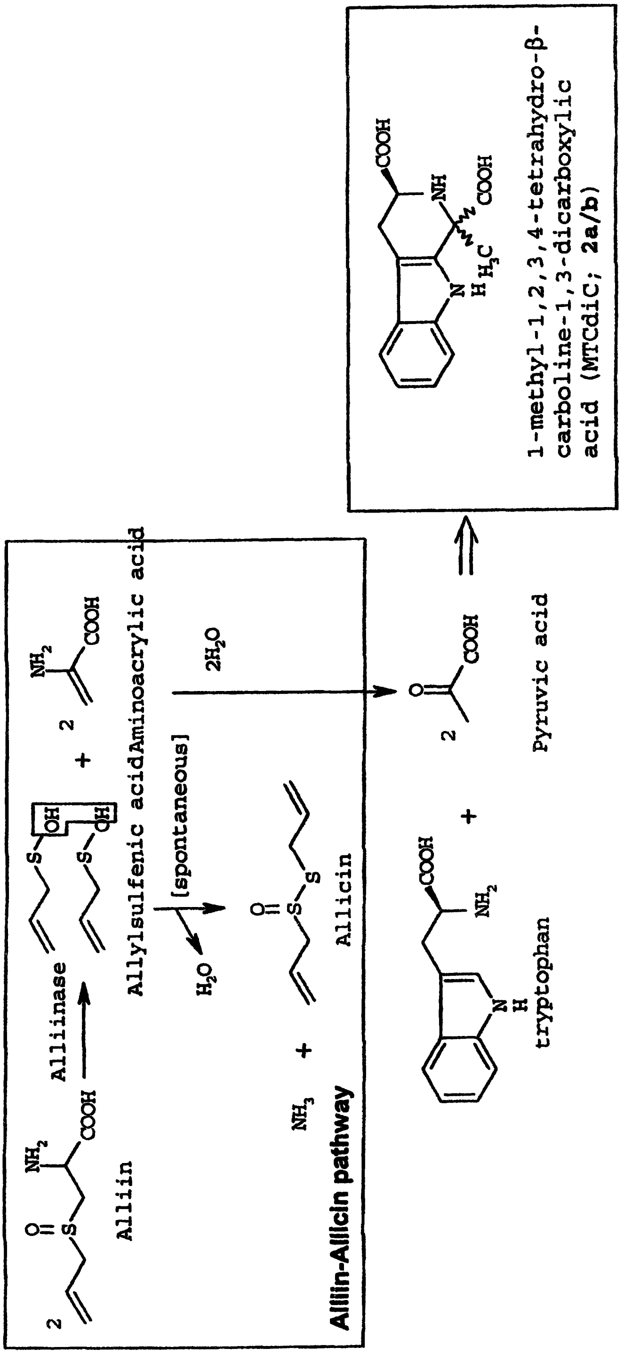
Alliin-Allicin pathway
2
Alliin
Alliinase
Allylsulfenic acid
Aminoacrylic acid
[spontaneous]
H2O
2H2O
NH3
Allicin
tryptophan
Pyruvic acid
1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (MTCdiC; 2a/b)

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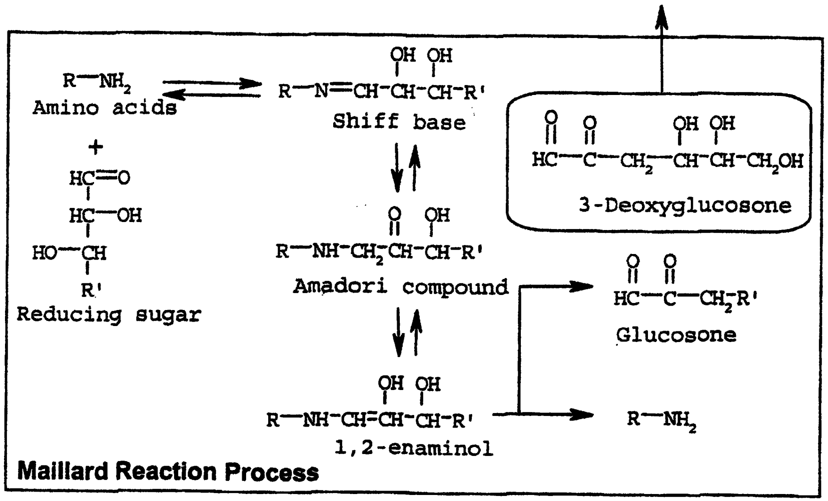
R—NH2
Amino acids
+
HC=O
HC—OH
HO—CH
R'
Reducing sugar
R—N=CH-CH-CH-R'
OH OH
Shiff base
R—NH-CH2-CH-CH-R'
O OH
Amadori compound
R—NH-CH=CH-CH-R'
OH OH
1,2-enaminol
HC—C—CH2—CH-CH-CH2OH
O O OH OH
3-Deoxyglucosone
HC—C—CH2-R'
O O
Glucosone
R—NH2
Maillard Reaction Process

Figure 7. Expected pathway to form 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (2a/b) during the natural aging process of garlic.

group at 1 position may explain the difference in scavenging activity between **1a/b** and **2a/b**. We also determined the effects of these compounds on AAPH-induced lipid peroxidation. We demonstrated that both (1S, 3S)-MTCdiC (**2b**) and (1S, 3S)-MTCC (**1b**) used in this system inhibited lipid peroxidation. It has been previously reported that in THβCs the saturated feature of the A ring provides the antioxidant activity (*7*). This feature may be considered as one of the mechanisms by which used THβCs inhibited lipid peroxidation.

The intracellular level of inducible nitric oxide synthase (iNOS) plays an important role in determining nitric oxide (NO) production rates in activated macrophages and several other cell types. In pathological condition, macrophages increase NO and superoxide anion productions resulting in the formation of peroxynitrite ($ONOO^-$), which can exert strong oxidant effects (*37, 38*). The high amounts of NO and/or $ONOO^-$ are associated with acute and chronic inflammation and atherosclerosis through cytotoxicity and injury to the surrounding cells and tissue. In this study, LPS-induced nitrite production in macrophages was measured. MTCdiCs (**2a/b**) exhibited inhibition at low concentrations. It was previously reported that aged garlic extract (AGE) inhibited LPS-induced NO production (*13*). Another group also reported that carboline derivatives isolated from *Melia azedarach* suppress iNOS activity through the inhibition of NF-κB activation (*39*). These data suggest that MTCdiCs (**2a/b**) identified in AGE may reduce the iNOS activity in activated macrophages and inhibit NO production. In conclusion, our data demonstrate that MTCCs (**1a/b**) and MTCdiCs (**2a/b**) in both diastereoisomers identified in AGE show potent antioxidant properties in *in vitro* systems. Especially, (1S, 3S)-MTCdiC (**2b**) is a more powerful hydrogen peroxide scavenger than the common antioxidant, ascorbic acid. Also, all of these compounds are not detected in raw garlic, but formed at the beginning of the natural aging process, and the contents increase during the aging. These data indicate that not only organosulfur compounds but also these alkaloids formed during the natural aging process may contribute to the antioxidant effects of AGE.

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Chapter 29

Tetrahydro-β-carboline Bioactive Alkaloids in Beverages and Foods

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Tetrahydro-β-carboline (THβCs) bioactive alkaloids occur in nutritional beverages and foods. Four of these compounds were identified by GC-MS and HPLC-MS in fruit juices and beverages: 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid, 1-methyl-1,2,3,4-tetrahydro-β-carboline and 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline. They were present in concentrations of up to several mg per liter in fruit juices and fermented alcoholic beverages. The formation of these alkaloids in beverages is via a nonenzymatic Pictet-Spengler condensation from indoleamines and aldehydes depending on the pH and temperature. Sulfur dioxide (SO_2) binds carbonyls, thus preventing the formation of THβCs alkaloids. The relatively abundant THβC-3-carboxylic acid are oxidized under heating, storage and oxidants such as nitrite, hydrogen peroxide and Fenton reagent to give the fully aromatic β-carbolines (βCs), norharman and harman. Beverages and foods containing THβCs and βCs are a significant source of these active compounds occurring in biological tissues and fluids.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

Tetrahydro-β-carbolines (THβCs) and β-carbolines (βCs) are naturally occurring indole alkaloids with a common tricyclic pyrido (3,4-b)indole ring structure. THβCs and βCs exhibit a broad range of pharmacological and biological activity (*1-3*). Biological interest in THβCs and βCs has grown from reports suggesting their occurrence under physiological conditions in biological tissues and fluids (*2,4-8*). These alkaloids have attracted the attention of neurochemists who have speculated on their putative role in the central nervous system where they might function as mild neuromodulators. They inhibit monoamine oxidase, monoamine uptake, and bind to benzodiazepine-GABA receptor (*2,3,7-10*). Simultaneously, THβCs and βCs have been studied in relation with alcoholism where they might play a role in the etiology or addiction, or in pathological states (*7,8,11-14*). β-carbolines are also interesting from a toxicological point of view because they could act as co-mutagens or precursors of mutagens (*15-17*), or be bioactivated to give endogenous neurotoxins (*18-20*). Taken together, a full delineation of the biological activity and possible toxicity of THβCs and βCs is still desirable and needed. In this regard, we have recently found that these compounds exhibit noticeable antioxidant activity (*21*).

Since tetrahydro-β-carboline and β-carboline alkaloids may exhibit biological actions, their availability during food consumption is of interest. These compounds may be formed under mild conditions in foods by a nonenzymatic Pictet-Spengler reaction (*22*). As a result they occur in commercial beverages and foods (*23-32*). A scheme illustrating the formation of THβC and βC heterocycles in foods and biological systems from indoleamine precursors is shown in Figure 1. Therefore, the diet might surely contribute to the ultimate presence of these alkaloids in the human biological tissues and fluids. The present paper focuses on the occurrence of tetrahydro-β-carbolines in commercial beverages such as fruit juices and fermented alcoholic beverages. We have identified several THβCs by GC-MS and HPLC-MS in many of those products and subsequently studied their occurrence by SPE-HPLC-fluorescence. In addition, the factors affecting the levels of THβCs produced by a Pictet-Spengler reaction during food production, processing and storage were studied, along with the possible formation of the fully aromatic β-carbolines (βCs) from THβCs as shown in Figure 1.

Experimental

Reference Compounds and Samples

1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) was prepared from L-tryptophan and acetaldehyde through Pictet-Spengler

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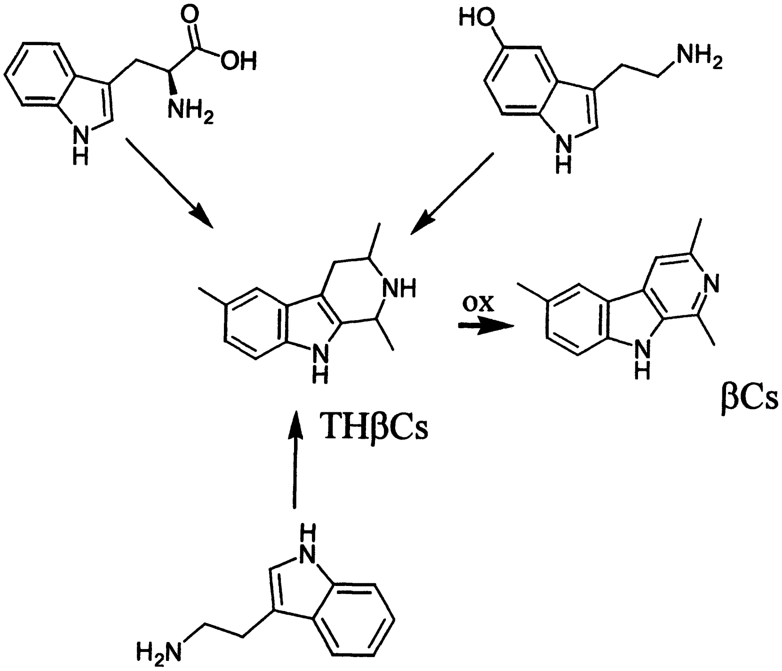

Figure 1. Tetrahydro-β-carbolines (THβC) produced from indoleamine precursors (tryptophan, serotonin and tryptamine) and their oxidation to give the fully aromatic β-carbolines (βC).

condensation (*33*), and also purchased from Sigma (Saint Louis MO). In the samé manner, 1-ethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (ETCA) (used as internal standard), and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) were prepared from L-tryptophan and propionaldehyde or formaldehyde, respectively. The compounds 1,2,3,4-Tetrahydro-β-carboline-1,3-dicarboxylic acid (THCA-COOH), and 1-methyl-1,2,3,4-tetrahydro-β-carboline-1,3-dicarboxylic acid (MTCA-COOH) were synthesized from L-tryptophan, and glyoxylic acid or pyruvic acid, respectively. 6-Hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline and 1-methyl-1,2,3,4-tetrahydro-β-carboline were obtained from the corresponding indoleamines and acetaldehyde through Pictet-Spengler reaction (*33*). Confirmation of the structures of the synthesized compounds was by NMR, MS, GC-MS, and RP-HPLC-MS (*33, 34*). The derivatives of *N*-methoxycarbonyl-tetrahydro-β-carboline-3-carboxylic acid methyl ester were obtained with methyl chloroformate in the presence of pyridine and methanol, or methyl chloroformate and diazomethane (*24*). Samples containing the standards or food-isolated tetrahydro-β-carboline-3-carbobylic acid (THβC-3-COOH) were derivatized to obtain the *N*-methoxycarbonyl esters and subsequently analyzed by GC-MS.

Commercial samples of nutritional beverages, fermented alcoholic beverages and foods, both from local and imported origin, were purchased from local supermarkets and subsequently used for the isolation and analysis of carboline alkaloids.

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Analysis of THβC-3-COOHs from Nutritional Beverages and Foods

THβC-3-COOHs were isolated by using SCX-solid phase extraction (*25, 27, 31*). Liquid samples such as fruit juices (20 mL) were added with 1mg/mL semicarbazide (Sigma), and centrifuged (5100g, 0-5°C) for 10-15 min. Solid samples were homogenized in 0.6 M $HClO_4$ using an ultraturrax and subsequently centrifuged. An aliquot of supernatant (5.5 mL) was spiked with 0.5 mL of ETCA solution (5 mg/L) used as internal standard (IS), acidified if needed with drops of 1M HCl, and slowly passed through benzenesulfonic acid SCX columns (Bond Elut, 500mg/3mL size, Varian, Harbor City, CA) using a vacuum manifold. Washing of SCX-columns was carried out with 0.1M HCl, methanol, phosphate buffer and THβC-3-COOHs eluted with phosphate buffer-methanol [*25*].

The analysis of THβC-3-COOHs was carried out using RP-HPLC and fluorescence detection (*23*). A 150 mm x 3.9 mm, 5 μm, Nova-pak C18 column (Waters, Milford, MA) was used for separation. Chromatographic conditions were as follows: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% of A in acetonitrile (buffer B). Gradient programmed from 0 (100% A) to 32% B in 8 min and then 90% B at 18 min. The flow rate was 1 mL/min, the column temperature was 40°C and the injection volume 20 μL. Fluorescent detection was set at 270 nm for excitation and 343 nm for emission.

Calibration curves from standard solutions of THβC-3-COOH were used for quantitation. Confirmation of the identity of THβC-3-COOHs was established by HPLC-MS and GC-MS. In addition, fluorescence spectra of HPLC peaks were compared with those of reference compounds. For this, eluting peaks corresponding to THβC-3-COOHs were trapped into the flow cell of the fluorescence detector by stopping the solvent pump, and excitation and emission spectra monitored (*35*).

GC-MS and HPLC-MS Analysis

THβC-3-COOHs were isolated from beverages and foods by solid phase extraction with SCX (benzenesulfonic silica) and C_{18} cartridges, and injected into GC-MS following derivatization (*24*). GC-MS analysis was performed onto a 20 m x 0.25mm methyl silicone capillary column by using a HP G1800A GCD system (GC-MS) working under electron ionization (EI). Oven temperature, 160°C (2 min), 4°C/min to 245°C (10 min); helium flow rate, 0.6 mL/min; injector temperature, 260°C; transfer line, 280°C, and ionization mode, 70 ev scanning from *m/z* 10 to 425.

Chemical identification of THβCs isolated by SCX was also accomplished by HPLC-MS on a 3.9 x 150 mm, 5 μm, Nova-pak C18 column, by using an HPLC-MSD series 1100 (Hewlett Packard) (electrospray-positive ion mode). Eluents: A: formic acid (0.5%); B: 0.5% formic acid in acetonitrile; 0-30% B in 30 min. Flow 0.5 ml/min. Cone voltage 50 V. Mass range 50-700 *amu*.

Formation of THβCs in Model Solutions and Beverages.

The formation of THβCs by a nonenzymatic Pictet-Spengler condensation was investigated from model reactions containing indoleamines (tryptophan 104 mg/L or tryptamine 80 mg/L) and aldehydes (formaldehyde, 50 mg/L or acetaldehyde, 48 mg/L) at different pH values (pH 1 of HCl and NaCl, and 50 mM phosphate buffer pH values: 3, 5, 7 and 9), temperatures (25, 37, 60 and 80 °C) and levels of sulfur dioxide (0, 87, 208, 417 and 833 mg/L added as $Na_2S_2O_5$). Additionally, beverages were spiked with exogenous formaldehyde or acetaldehyde and further determining the increase of THβC-3-COOHs (23, 25). For that, test tubes containing 10 mL of orange juice were separately added with acetaldehyde at zero (control), 20, 50 and 100 mg/L, or formaldehyde at zero (control), 20, 50 and 100 mg/L and kept for 48 h at 30 °C.

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Oxidation of Tetrahydro-β-carbolines to Afford β-Carbolines (βCs)

Standard solutions of tetrahydro-β-carbolines (THβCs) were prepared in 100 mM phosphate buffer pH 4 as follows: 50 μM THCA, 50 μM MTCA, 50 μM 1,2,3,4-tetrahydro-β-carboline, 50 μM 1-methyl-1,2,3,4-tetrahydro-β-carboline, 50 μM MTCA-COOH and 50 μM THCA-COOH. Subsequently, the model solutions were heated at 60 and 80 °C for 1 and 3 h, or treated separately with a variety of oxidants such as H_2O_2 (2 mM), $NaNO_2$ (100 μM), H_2O_2 (50 μM) + $FeSO_4$ (50 μM), $FeCl_3$ (1 mM). Then, the concentration of the aromatic β-carbolines, norharman and harman, that appeared into the model solution was determined by RP-HPLC-fluorescence. Chromatographic analysis was accomplished as for THβC-3-COOHs by changing the fluorescence detection at 300 nm for excitation and 433 nm for emission (*35-37*).

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Results and Discussion

Chemical Identification of THβCs in Beverages and Foods

In order to accomplish the chemical identification of tetrahydro-β-carboline (THβC) alkaloids in foods by GC-MS, the electron ionization (EI)-mass fragmentation pattern of THβC alkaloids and their precursors tryptamine and tryptophan, both as free and derivatized species, including *N*-trifluoroacetyl and *N*-methoxycarbonyl derivatives, were studied (*33*). Fragmentation of THβCs was dominated by retro Diels-Alder rearrangement (-73 *amu*) which afforded the fragment m/z 143 as base peak for tetrahydro-β-carbolines, and the ion *m/z* 157 for 1-methyl-tetrahydro-β-carbolines (Figure 2). In addition, THβCs provided abundant molecular ions. The loss of substituents at C-1 position of the tetrahydro-pyrido ring and groups at the *N*-pyrido and 3-carboxylates ($COOCH_3$ or $COOCH_2CH_3$) were also characteristic of these compounds (*33*). Volatile THβC-*N*-methoxycarbonyl methyl ester derivatives amenable for GC-MS analysis were obtained by reacting the THβCs with methyl chloroformate in the presence of pyridine and methanol, or alternatively methyl chloroformate and diazomethane as shown in Figure 2 (*24,33*). Taken advantage of this derivatization method, two THβC-3-COOHs (MTCA and THCA) and 1-methyl-tetrahydro-β-carboline were positively identified in beverages and foods by GC-MS. THβC alkaloids were found in many beverages and foodstuffs such as wine, beer, wine vinegar, fruit juices, apple cider, soy and Tabasco sauces, blue cheese, yogurt and toasted bread (Table I). Figure 3 shows a GC-MS chromatogram corresponding to THβC-3-COOHs in an orange juice. 1,3-Disubstituted tetrahydro-β-carbolines such as 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) occurred as two diastereoisomers of

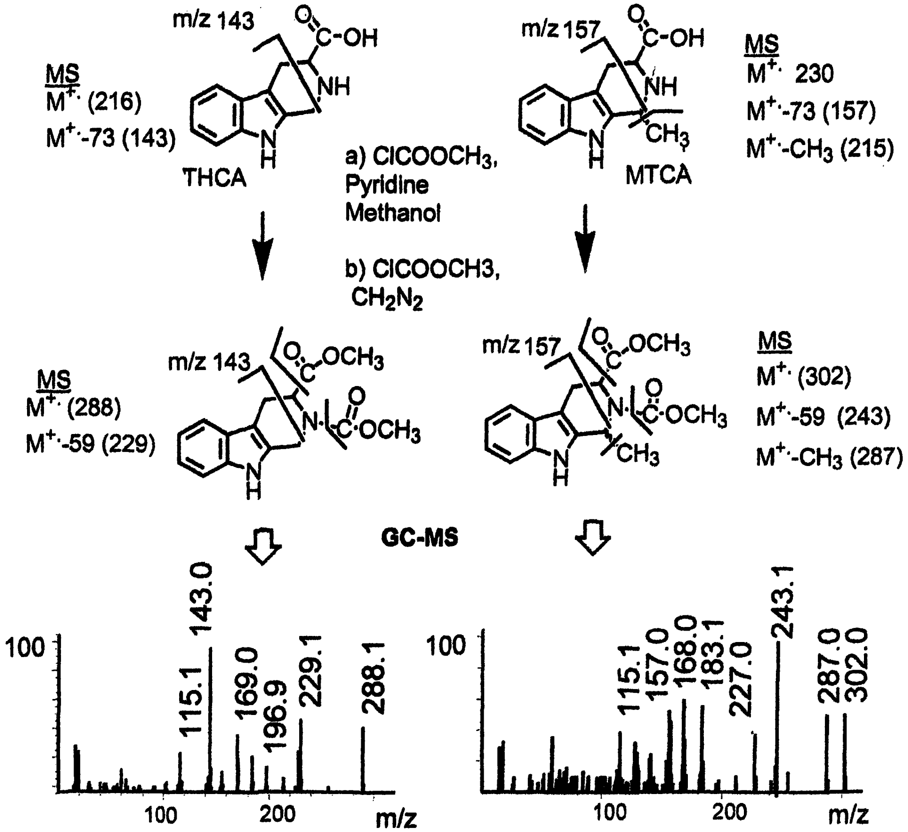
MS
M+· (216)
M+·-73 (143)
m/z 143
C-OH
NH
THCA
a) ClCOOCH3, Pyridine Methanol
b) ClCOOCH3, CH2N2
m/z 157
MTCA
CH3
MS
M+· 230
M+·-73 (157)
M+·-CH3 (215)
MS
M+· (288)
M+·-59 (229)
OCH3
C-OCH3
MS
M+· (302)
M+·-59 (243)
M+·-CH3 (287)
GC-MS
100
115.1
143.0
169.0
196.9
229.1
288.1
100
200
m/z
115.1
157.0
168.0
183.1
227.0
243.1
287.0
302.0
m/z

Figure 2. Electron Ionization mass fragmentation pattern of tetrahydro-β-carbolines (THβC) and their N-methoxycarbonyl methyl ester derivatives used for GC-MS identification in beverages and foods.

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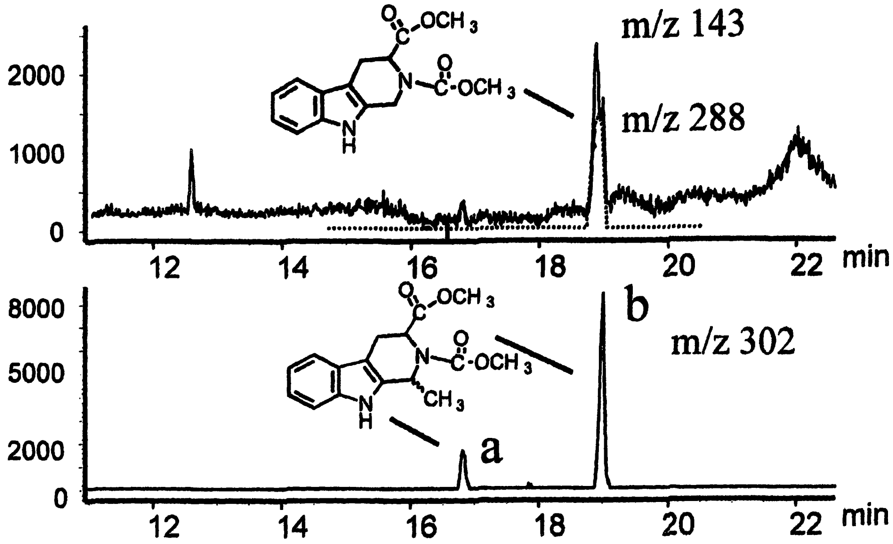

Figure 3. Selected ion chromatogram of THβC-3-COOHs in orange juice by GC-MS as N-methoxycarbonyl methyl ester derivatives. MTCA appears as two diastereoisomers (a, b).

configuration 1*S*,3*S* and 1*R*,3*S*, that were separated by the GC-MS affording similar EI mass spectra.

Table I. THβC-3-COOHs identified in beverages and foods by GC-MS as *N*-methoxycarbonylmethyl ester derivatives[1].

	THβC-3-COOH		
	1R,3S-MTCA	*1S,3S-MTCA*	*THCA*
Wine	+	+	-
Beer	+	+	+
Cider	+	+	+
Wine vinegar	+	+	+
Soy sauce	+	+	+
Tabasco sauce	+	+	+
Orange juice	+	+	+
Toasted bread	+	+	+
Blue cheese	+	+	+
Yogurt	+	+	(+)

[1] 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA; diastereoisomers 1*S*,3*S*, major compound and 1*R*,3*S*, minor compound) and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA).

Source: Reproduced from ref (*24*) with permission. Copyright 1997. Elsevier Science

In a further step, the chemical identification of THβCs in nutritional beverages and foods by HPLC-MS (electrospray ionization) was carried out. After the corresponding sample preparation by solid phase extraction, these compounds were analyzed by RP-HPLC-MS without previous chemical derivatization. SCX-extracted samples of fruit juices gave trace ions and spectra corresponding to THβCs (Figure 4). Electrospray ionization afforded good protonated molecular ions allowing identification of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline at *m/z* 203 (M+H)$^+$, 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) at *m/z* 217 (M+H)$^+$, 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid as two diastereoisomers 1*S*,3*S*-MTCA and 1*R*,3*S*-MTCA at *m/z* 231 (M+H)$^+$, and 1-methyl-1,2,3,4-tetrahydro-β-carboline at *m/z* 187 (M+H)$^+$ in fruit juices. The relative presence and concentration of alkaloids depended on the type of fruit juice. Thus, citrus fruit juices showed a high level of MTCA, whereas tropical fruit juices such as pineapple juice contained a high level of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro-β-carboline.

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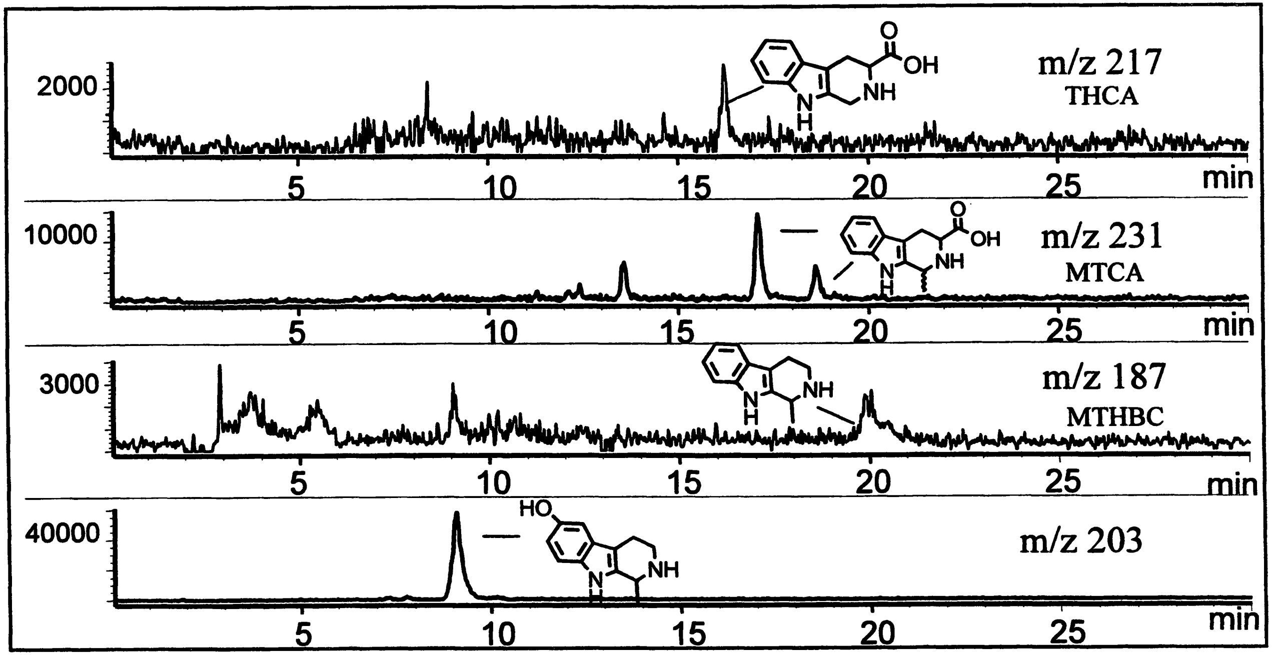

Figure 4. THβCs identified in commercial multifruit juice by HPLC-MS (electrospray ionization).

Occurrence of Tryptophan-derived THβC-3-COOHs in Beverages

Among the tetrahydro-β-carbolines (THβCs) identified above, we have initially focused on two tryptophan-derived tetrahydro-β-carboline-3-carbobylic acid (THβC-3-COOHs) such as MTCA and THCA. These compounds were isolated from juices and beverages by solid-phase extraction and analyzed by HPLC-fluorescence detection (*23,27*). Figure 5a illustrates the concentration of THβC-3-COOHs found in several alcoholic and nutritional beverages; among samples examined the highest content was detected in fermented alcoholic beverages such as wines, high alcohol beers, and fruit liquors. Fruit juices contained a relatively high amount of THβC-3-COOHs, particularly MTCA. Minor amounts were found in yogurt specially those containing fruits or fruit juices, whereas only traces were detected in soft drinks containing fruit derived ingredients. Figure 5b shows the level of tryptophan-derived alkaloids found in several fruit juices. Citrus fruit juices such as those from orange and grapefruit contained the highest amount of THβC-3-COOH along with multifruit, grape and vegetable juices like those from tomato. The rest of juices contained a much lower amount of these compounds although they appeared in most of them. Generally MTCA was the major tetrahydro-β-carboline, although exceptionally some vegetable juices seemed to contain a high level of THCA. Other THβCs detected by mass spectrometry, as mentioned above, such as 1-methyl-tetrahydro-β-carboline, and 6-hydroxy-1-methyl-tetrahydro-β-carboline were not further quantified here, though they appeared in some fruit juices in concentrations as high as those of THβC-3-COOH (THCA and MTCA).

Formation of Tetrahydro-β-carbolines (THβCs) in foods

From preparative synthesis, it is generally assumed that THβCs are produced from indoleamines and aldehydes or α-ketoacids through Pictet-Spengler condensation. However, not much has been done to rationalize how chemical, technological or biological factors in foods may affect this reaction (*22*). We have studied the effect of pH, temperature, SO_2 and fermentation during winemaking. Although this reaction is rather feasible in the presence of reactants, the pH of the media is a determinant factor of the reaction rate (Figure 6). Indeed, in tryptophan-acetaldehyde carbolines the reaction at *quasi*-physiological pH was very slow, whereas being fast at low pH. At the pH of many beverages and foods (pH values 3-4), this reaction progressed readily to afford the corresponding THβCs (Figure 7). The condensation of tryptophan with formaldehyde to give THCA, however, occurred in a much wider pH interval, including neutral pH. Reaction with formaldehyde was faster and less pH-dependent than acetetaldehyde. As expected, the reaction rate of this

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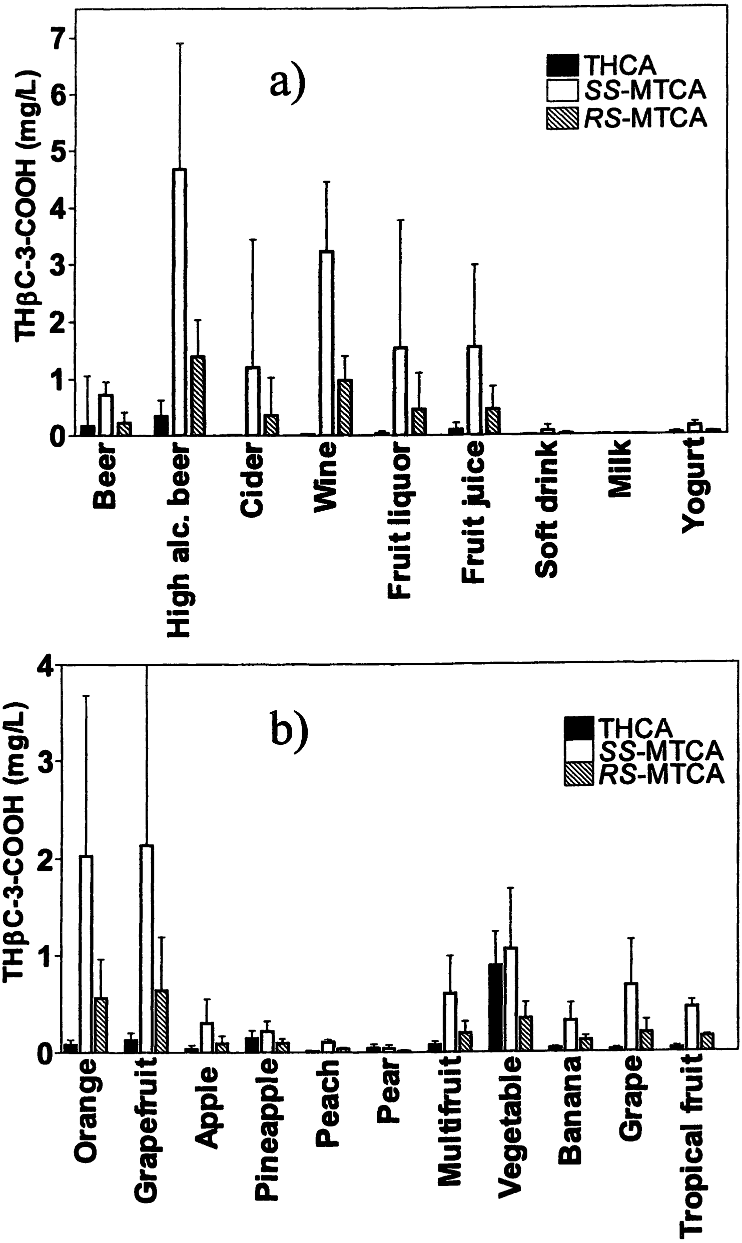

Figure 5. THβC-3-COOHs in beverages (a) and fruit juices (b). 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA, as 1S,3S and 1R,3S diastereoisomers).

condensation increased with temperature, regardless of the pH of the media (Figure 6c). As the temperature increased, the ratio of MTCA diastereoisomers (*SS* to *RS*) decreased owing to the involvement of thermodynamic control versus kinetic control into the Pictet-Spengler reaction.

By sequestering carbonyls involved in the reaction (i.e. aldehydes or α-ketoacids), the formation of THβCs (*22*) could be decreased. Sulfur dioxide (SO_2), a common antioxidant and preservative in beverages and foods, efficiently prevented or, at least, highly decreased the formation of THβCs (Figure 6d). This fact is mainly due to the removal of free aldehydes by reaction with SO_2 to afford hydroxysulfonate adducts. These results might be of interest if seeking to reduce the presence of THβCs in foods. SO_2 exhibited the same effect during the alcoholic fermentation of grape must in winemaking, that is generally accomplished in the presence of sulfur dioxide (Figure 8) (*22*). The formation of MTCA during the alcoholic fermentation by yeast paralleled the formation of acetaldehyde, probably as this carbonylic compound condensed with tryptophan. Then, the fermentation process was a significant factor determining the level of MTCA into the wine.

The above results leads to the conclusion that a Pictet-Spengler reaction providing THβCs proceeds in foods and probably in biological systems as demonstrated for the formation of tetrahydro-β-carboline-3-carboxylic acids (THβC-3-COOHs) from L-tryptophan and formaldehyde or acetaldehyde. It is well known that these aldehydes are present in many foods and beverages in variable amounts (*38*). A proposed scheme for the formation of THCA and MTCA in foods from tryptophan and aldehydes is shown in Figure 9. Alternatively, THβC-3-COOH might come from the corresponding 1,3-dicarboxylic-THβC alkaloids by decarboxylation in acidic media. The latter compounds have also been reported in some foods (*29*), and might be derived from condensation of tryptophan with glyoxilic acid and pyruvic acid in acidic media. The content of THβC-3-COOHs may be determined by the relative presence of aldehydes in foods. Thus, MTCA (1*S*,3*S* and 1*R*,3*S* diastereoisomers) was generally the major carboline in beverages because of the presence of the highest amount of free acetaldehyde as compared to formaldehyde in them.

Formation of β-carbolines (βCs) from Tetrahydro-β-carbolines (THβCs)

The fully aromatic β-carbolines (βCs) constitute a particular subgroup of indole-derived alkaloids. Two of these compounds, norharman and harman, might exhibit potential biological activity as psychoactive and/or co-mutagenic substances (*3, 17, 37*). βCs have been reported in well-cooked foods, and alcoholic beverages (*36, 37, 39*), and recently in coffee brews (*40*). Their concentration is usually in the order of ng per g level (ng/g), so that much lower

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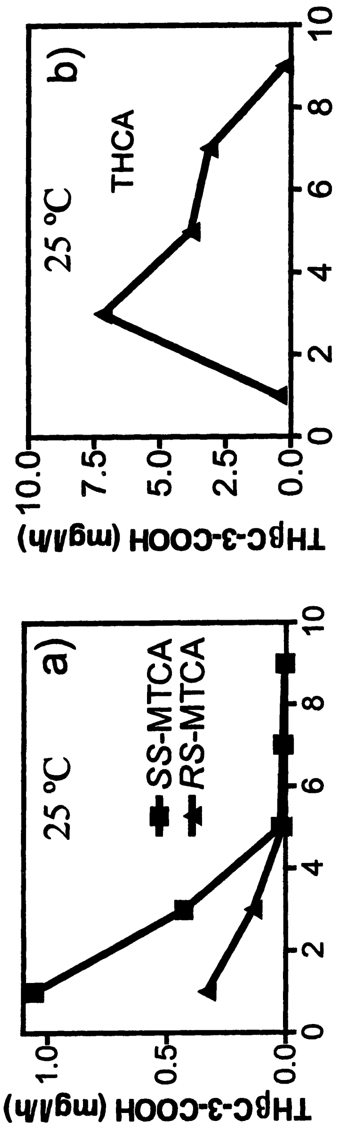

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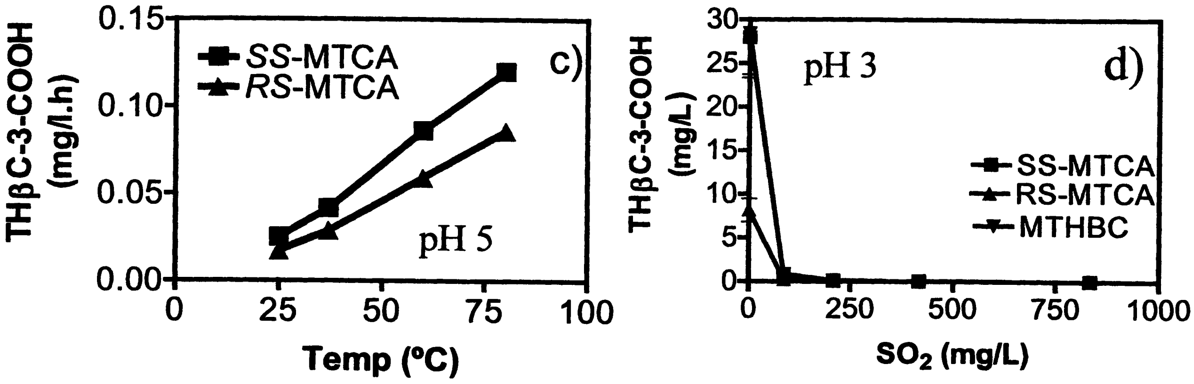

Figure 6. Pictet-Spengler condensation to give THβCs from indoleamine and aldehydes. Acetaldehyde, 48 mg/L (a and c) or formaldehyde, 50 mg/L (b) reacted with tryptophan (104 mg/L) at various pH values or temperatures. Reaction rate (mg/L/h) calculated during the first 4 h. Acetaldehyde (47 mg/L) and indoleamines (tryptophan, 52 mg/L and tryptamine, 40 mg/L) reacted in presence or absence of SO_2 for 10 days at pH 3 to give MTCA and MTHβC, (1-methyl-tetrahydro-β-carboline) (d).

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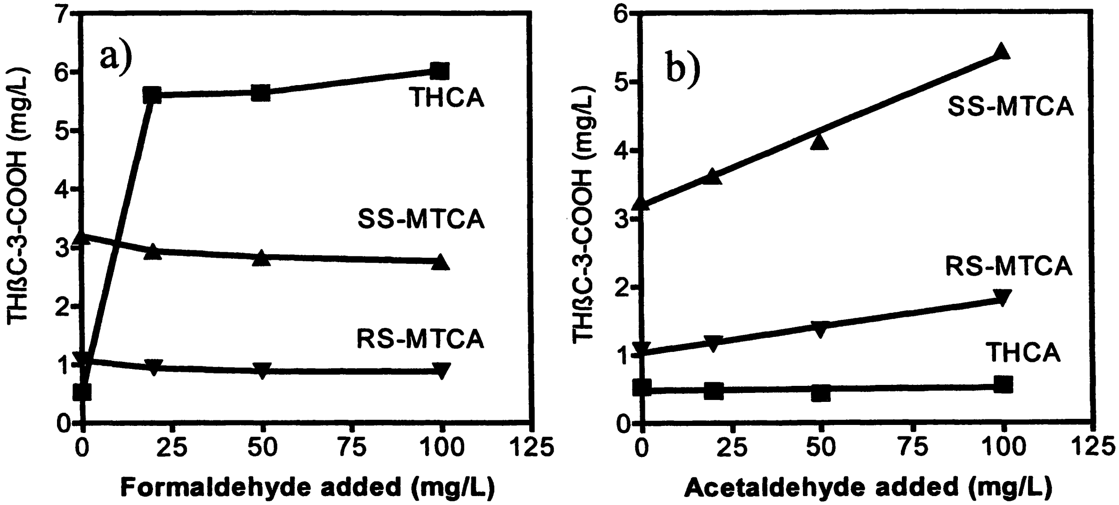

Figure 7. Formation of THβC-3-COOHs in orange juice spiked with formaldehyde or acetaldehyde. Adapted from (23). Copyright 1996. ACS.

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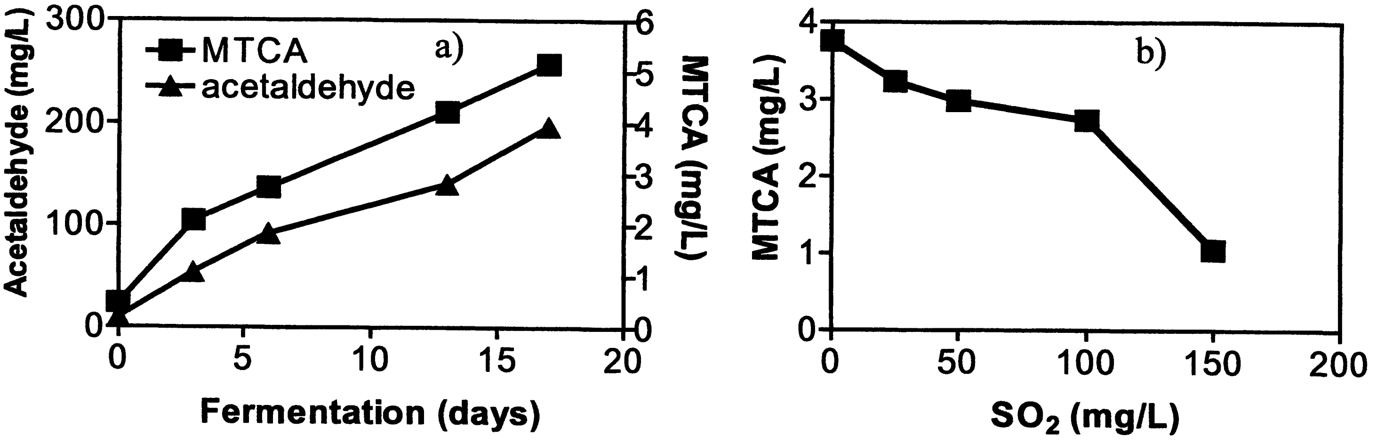

Figure 8. THβC-3-COOHs (MTCA) and acetaldehyde produced during the alcoholic fermentation of a Chardonnay grape must in winemaking (a). MTCA into the final wine versus the level SO_2 adjusted before fermentation (b). Adapted from (22). Copyright 1993, ACS.

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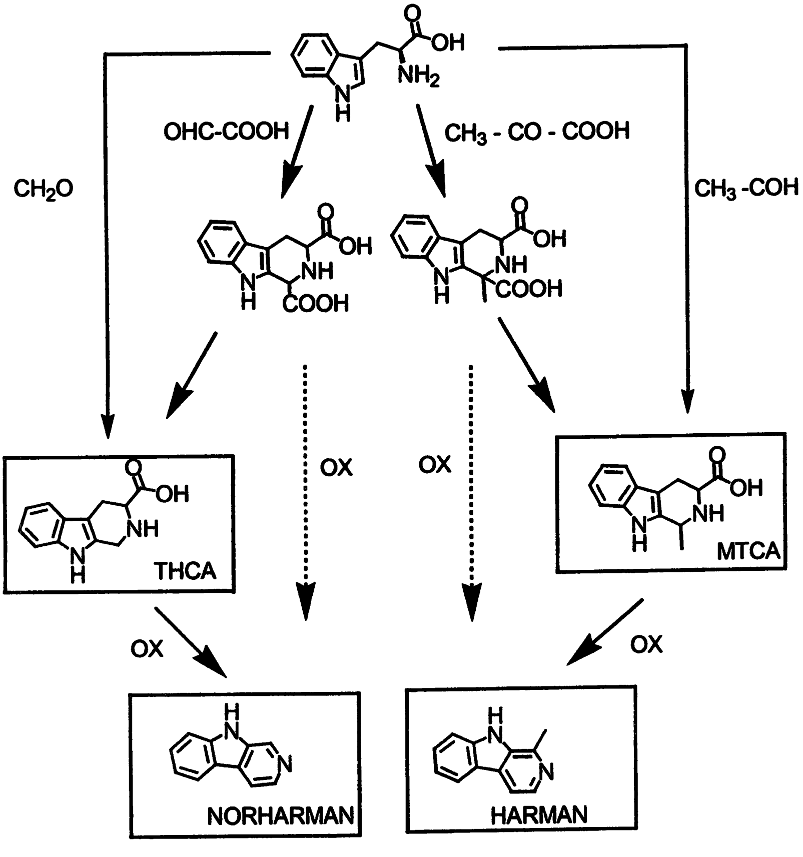
OH
NH2
N H
OHC-COOH
CH3 - CO - COOH
CH2O
CH3 -COH
OH
NH
COOH
OX
OX
THCA
MTCA
OX
OX
NORHARMAN
HARMAN

Figure 9. Formation of THβC-3-COOHs in beverages and foods by Pictet-Spengler reaction. Oxidative decarboxylation of THβC-3-carboxylic acid or THβC-1,3-dicarboxylic acid gives the fully aromatic βCs.

than THβCs. The origin of these alkaloids is likely the amino acid tryptophan but the actual way to them is unknown. Two possible pathways are expected: one from the corresponding THβC-3-COOHs and the other through THβCs themselves. It has previously been suggested that βCs in foods might come from THβC-3-COOH (*36*). As showed in Figure 10, the relatively abundance of THβC-3-COOHs is likely the actual precursor of βCs in beverages and foods. Several factors such as heating, presence of oxidants such as hydrogen peroxide, hydrogen peroxide and transition metals (Fe^{2+}) (Fenton reaction), sodium nitrite and even storage at room temperature accelerated the formation of the βCs norharman and harman from the corresponding THβC-3-COOHs. Under the same conditions, tetrahydro-β-carbolines (THβCs) lacking a 3-carboxylic acid did not provide any appreciable amount of βCs, whereas tetrahydro-β-carboline-1,3-dicarboxylic acids also afforded minor amounts of βCs at the highest temperature of heating (80 °C) and in the presence of oxidants (results not shown). Therefore, the fully aromatic βCs produced in foods, food processing or cooking may come from the corresponding THβC-3-COOHs through decarboxylative oxidation that may occur even under mild conditions. As the βCs norharman and harman occur in rather low amounts in foods and beverages, a mild and limited oxidation of the more abundant THβC-3-COOH, or alternatively the THβC-1,3-dicarboxylic acids, is likely to afford those alkaloids (see Figure 9).

Overview and Conclusions

The above results show the occurrence of THβCs alkaloids in food, nutritional fruit juices and fermented alcoholic beverages. Several alkaloids were identified in such samples both by GC-MS (electron ionization) and HPLC-MS (electrospray ionization). The occurrence of THβCs in other nutritional foodstuffs such as chocolate has also been reported (*41*). In samples considered here, the highest concentration of tryptophan-derived alkaloids was in fermented alcoholic beverages such as wine and beer, and in a variety of fruit juices and fruit liquors. It is assumed that THβCs may occur as a Pictet-Spengler condensation from indoleamines and aldehydes or α-ketoacids. Experimental results showed that acetaldehyde and formaldehyde reacted with tryptophan under mild conditions to provide THβC-3-COOHs both in foods and model systems. The reaction rate was dependent on the pH and temperature. The presence of aldehyde-binding compounds such as sulfur dioxide highly reduced or even prevented the formation of these alkaloids.

A particular subgroup of these carboline alkaloids is the fully aromatic βCs norharman and harman. These compounds are produced from tetrahydro-β-carboline-3-carboxylic acid through oxidative decarboxylation. This was the

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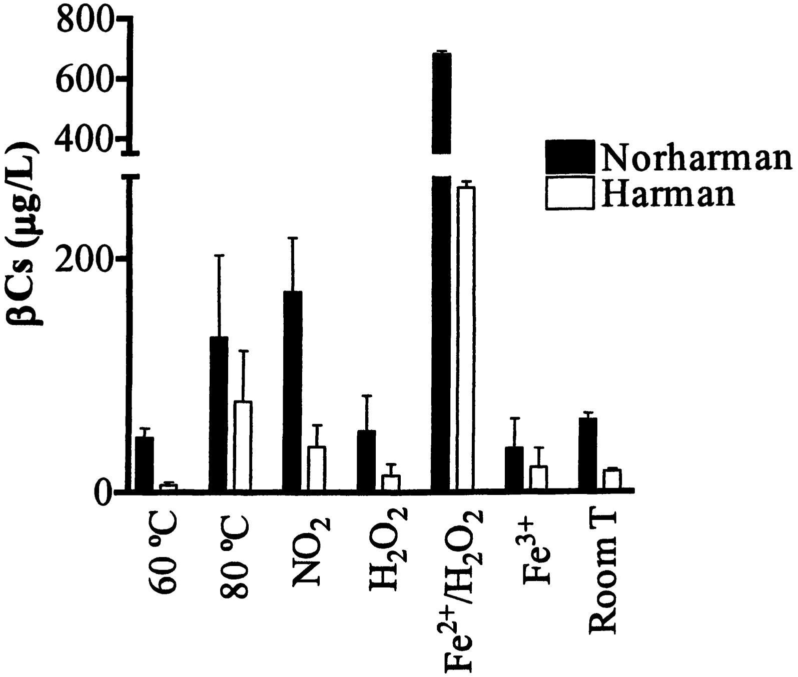

***Figure 10.** Norharman and harman βCs produced from the corresponding THβC-3-COOHs (THCA and MTCA) in different conditions (60 and 80 C, 3h; 100 µM $NaNO_2$, 1h; 2 mM H_2O_2, 1h; 50 µM Fe^{2+}/ H_2O_2, 1h; 1mM Fe^{3+}, 24 h and storage at room T, 7 days).*

case during heating, storage and in presence of oxidants. It is expected that many technological factors during elaboration, processing or storage may contribute to the final concentration of βCs in foodstuffs.

THβCs have been detected in biological tissues and fluids and such mammalian alkaloids may exhibit biological actions. Interestingly, we have recently found that they exhibit noticeable antioxidant activity (*21*). The results presented in this communication suggest that the diet via foods containing THβC alkaloids represents an important exogenous source of these compounds. THβCs might hypothetically accumulate into tissues and fluids. So, although *in vivo* endogenous formation itself should not be ruled out, we should conclude that the presence of THβCs in the human body might partially arise from beverages and food intake.

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Chapter 30

Influence of DNA on Volatile Generation from Maillard Reaction of Cysteine and Ribose

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The effects of DNA on thermal flavor generation were investigated using the Maillard reaction model system containing cysteine and ribose, which were heated to the roasting temperature of 180 °C for 2 hours at pH 5 and pH 8.5. The volatiles and semi-volatile compounds identified from the liquid phase of the reaction systems of ribose and cysteine with or without DNA indicated that DNA affected volatile formation in a complex manner. A typical compound derived from DNA-involved thermal reaction was 2-furfuryl alcohol, which elicits a sugary aroma. DNA mitigated the sulfurous, meaty aroma by reducing the concentration of some well-known meaty flavor compounds such as 2-methyl-3-furanthiol, 2-methyl-3-thiophenethiol, 2-furfurylthiol and their associated dimers as well as some thiophenes. On the other hand, DNA promoted the formation of several important nitrogen-containing volatiles such as methylpyrazine, 2-acetylthiazole, cyclopentapyrazine and 2,6-dimethylpyrazine, which are known to elicit roasty, nutty flavor notes. Although DNA can act as a nitrogenous source, it should not be regarded as a major donor in the formation of nitrogen-containing compounds.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

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A number of studies utilizing various analytical techniques in recent years have revealed some very important aroma compounds imparting meat flavor perception (*1-3*). Out of more than 1000 volatiles identified from cooked meats, a few have been characterized as meaty flavor impact compounds. In general, thiol-substituted furans and thiophenes and related disulfides possess strong meat-like and/or roast aromas with low odor threshold values. For instances, 2-methyl-3-furanthiol and its disulfide bis-(2-methyl-3-furyl)-disulfide were identified as major contributors to the meaty aroma of cooked beef, chicken, and pork (*4,5)* while 2-furfurylthiol was identified as a contributor to roasty and coffee-like notes (*6*).

It is well known that the Maillard reaction between reducing sugars and amino acids is one of the crucial routes for the formation of these compounds during cooking. Studies have shown that cysteine and pentoses are important precursors participating in the thermal reaction to generate meaty aroma compounds. In meats ribose is one of the major sugars, and it originates principally from ribonucleotides, in particular, adenosine triphosphate (*7*). Postmortem effects result in a large amount of nucleotides from degradation of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

Besides serving as the source for pentose sugar, nucleotides have been found to act synergistically with glutamic acid or monosodium glutamate (MSG) to enhance meaty, brothy, and MSG-like taste while suppressing sulfurous notes (*8*). Two nucleotides, inosine-5'-monophosphate (IMP) and guanosine-5'-monophosphate (GMP), which accumulate in post-slaughter muscle as a result of the hydrolysis of inosine triphosphate and guanosine triphosphate, respectively (*1*), are taste active. They both have been used as flavor enhancers in savory foods and are believed to contribute to the "umami" taste (*9*).

Thermally, these nucleotides are not stable. Their contents can be decreased by half within approximately 30 min at 121 °C in highly acidic conditions (*10*). The thermal degradability of these nucleotides makes them potential modifiers of thermally generated flavors. However, previous studies related to the influence of nucleic acids on meaty flavor generation have primarily focused on IMP. In a meat model system, IMP enhanced the formation of thiols and novel disulfides containing furan moieties (*1*). Volatiles generated from the reaction of both alliin-IMP and deoxyalliin-IMP elicited a pungent garlic flavor with roasted notes, caused by sulfur-containing compounds plus a number of pyrazines and thiazoles, including methylpyrazine, ethylpyrazine, 2,5-dimethylpyrazine, 2-propylthiazole, and 2-ethyl-4-propylthiazole (*11*).

The level of IMP was 0.106-0.443% in beef and 0.075-0.122% in chicken; chicken appeared to contain a somewhat higher amount of AMP, CMP, UMP than beef (*10*). It was reported that the normal concentration of DNA in longisimus muscle was about 1091.9 μg/g muscle (*12*). The high level of DNA in meats potentially has some effects on meat flavor production during cooking. However, information regarding the overall effect of DNA on meaty flavor is virtually unavailable. The influence of DNA on the formation of volatile compounds at both pH 5 and 8.5 using a Maillard reaction model system of ribose and cysteine was examined in this study.

Experimental

Materials

D-ribose, tridecane and anhydrous sodium sulfate were purchased from Aldrich Chemical Company (Milwaukee, WI). DNA and L-cysteine were bought from Sigma Chemical Co. (St. Louis, MO). Methylene chloride used was of HPLC grade and purchased from Fisher Scientific (Fair Lawn, NJ).

Thermal Reactions

The powdered DNA was made from Herring sperm and had a fishy odor. For each reaction involving DNA, 3 g of DNA powder was weighted and thermally dissolved in distilled water. The solution was extracted with 120 ml (40 x 3) of hexane to remove possible lipid residues. L-cysteine and D-ribose (0.01 mol) with or without 3 g of DNA were dissolved in 100 ml of distilled water. The addition of DNA into the solution of ribose and cysteine lowered the pH by about 1 unit. The pH of solutions was adjusted to 5.0 or 8.5 with sodium hydroxide prior to thermal reaction in 150 ml Hoke stainless steel cylinders (Hoke Inc., Clifton, NJ), which were heated at 180 °C in an oven for 2 h. The reactions were immediately stopped by cooling under a stream of cold water. Other thermal reactions of DNA, between DNA and ribose or cysteine were all conducted in a similar manner and under the same conditions.

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Liquid/liquid Extraction of Volatile/Semivolatile Compounds

After cooling, the brown reaction mixture was mixed with 0.5 ml of a solution of internal standard (tridecane, 1 mg/ml) and extracted with methylene chloride (50 ml x 3 times). The extract was dehydrated by anhydrous sodium sulfate and concentrated under a nitrogen flow to 10 ml in a flask and then transferred to a Kuderna-Danish concentrator and further concentrated to 1-1.5 ml before subjecting to further analysis.

GC/Mass Spectrometry Analysis

The concentrated isolates from different reaction mixtures were analyzed by GC/mass spectrometry (GC/MS), using a Hewlett-Packard 6890 GC equipped with a fused silica capillary column (60 m x 0.25 mm i.d.; 1 μm thickness, DB-1) coupled to a Hewlett-Packard 5973 series mass selective detector. Mass spectra were obtained by electron ionization at 70 eV and a source temperature of 250 °C.

Identification of the Volatiles

The identification of volatile compounds was based on GC/MS analysis. The compounds from the isolate were identified by comparing the mass spectral data with those of authentic compounds available in the Wiley 275 library or previous publications (*2,13-15*). Quantification of the volatile/semivolatile compounds from the liquid phases was based on using tridecane as an internal standard.

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Results and Discussion

The effect of DNA on volatile generation was investigated using Maillard reaction solutions of ribose and cysteine with or without DNA at both starting pH of 5 and 8.5, which were heated at roasting temperature of 180 °C for 2 h. As indicated by Table I, pH changed 2-3 units after the reactions. Changes of pH are common in the reaction systems in the absence of buffering agents (*16*).

Table I. Final pH, final appearance and flavor description of the thermal reaction mixtures of ribose and cysteine with/without DNA

Solutions of Model Systems	Initial pH	Final pH	Aroma Characteristic
DNA	5	4.27	Fruity, sugary
	8.5	5.55	Slightly burnt
DNA + Ribose	8	3.61	Sugary
	8.5	4.25	Sugary, fruity
Ribose + Cysteine	5	3.56	Sulfurous, meaty
	8.5	5.25	Sulfurous, burnt, nutty
Cysteine + DNA	5	5.31	Sulfurous
	8.5	7.08	Sulfurous, burnt
Ribose + Cysteine + DNA	5	3.67	Sulfurous, meaty
	8.5	5.42	Nutty, burnt

Organoleptically, the odor elicited from the reaction of ribose and cysteine under acidic conditions was sulfurous and meaty, but under basic conditions, the odor had additional roasty and burnt notes, and the sulfurous, meaty aroma

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became less obtrusive. The presence of DNA in the reaction systems imparted some sugary, fruity, and sweet notes in addition to the aroma characteristics perceived in the absence of DNA.

Compounds identified from the liquid phases of the reactions between ribose and cysteine with or without DNA are listed on Table II. The data provided clear evidence that DNA can act as a major source for pentose. The addition of DNA increased the levels of furans, especially 2-furfuryl alcohol, furfural and difurylmethane. In the reaction systems without DNA, 2-furfurylalcohol was formed in small quantities but addition of DNA increased its concentration by many folds. Believed to have a fruity and sugary aroma, 2-furfuryl alcohol has been identified as a major component from baked sweet potato (*17*) and rice cake (*18*). It can also be generated in large amounts from the reaction between cysteine and glucose (*19*) and found to have strong inhibitory ability toward hexanal oxidation in commercial beer (*20*). Furfural serves as an important precursor for the formation of other furanoids and heterocyclic compounds such as thiophenes and pyrroles. When H_2S or NH_3 are present, replacement of the oxygen on the furan ring with sulfur or nitrogen results in the formation of the corresponding thiophenes or pyrrole derivatives.

Consistent with previous research reports (*1,21,22*), the results indicated that more sulfur compounds were produced at lower pH. The meaty flavor impact compound 2-methyl-3-furanthiol and its oxidized dimer bis (2-methyl-3-furyl) disulfide were formed in a much larger amount under acidic conditions and DNA appeared to inhibit their formation. These two compounds have the potency to dictate the characteristics flavor of meat. It has been shown that compounds containing a 2-methyl-3-furanyl group could originate from the reaction of hydrogen sulfide with 4-hydroxy-5-methyl-3(2H)-furanone (HMF) (*23*), which may derive from pentoses in the Maillard reaction or from the dephosphorylation of ribose phosphate (*16,24*). It is well known that formation of HMF from pentose sugar is favored under more acidic conditions. A significant amount of 2-furfurylthiol was generated from reaction of ribose with cysteine at pH 5, much less formed at pH 8.5. This is in accordance with previous studies showing that 2-furfurylthiol formation was greatly affected by pH and lowering the pH would significantly increase its formation (*25*). The formation of 2-furfurylthiol probably resulted from the reaction of H_2S with the ribose breakdown product furfural (*23*). Although DNA increased the amount of furfural, it did not show the same effect on 2-furfurylthiol formation. Instead, it greatly reduced the quantity of 2-furfurylthiol and its corresponding dimer, *bis* (2-furfuryl) disulfide. Possessing a characteristic coffee-like aroma, 2-furfurylthiol has been identified from the reaction between cysteine and inosine 5'-monophosphate (*26*).

The thiophenoids were the most numerous compounds identified. Among them, 2-methylthiophene was the most abundant, followed by thieno[3,2-b] thiophene and 5-methylthieno[2,3-d] thiophene. In general, addition of DNA

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Table II. Volatile Compounds Generated from the Liquid Phases of the Thermal Reactions of Ribose and Cysteine, with/without DNA at pH 5 or pH 8.5

	Amount (mg / g ribose)			
Compounds	**RC 5^a**	**RCD 5^b**	**RC 8.5^c**	**RCD 8.5^d**
Furanoids				
Furfural	0.172	0.487	0.011	0.023
2/3-Furfuryl alcohol	0.023	1.471	0.027.	5.021
2-Acetyl-5-methylfuran		0.013		
Difurylmethane	0.006	0.247		0.049
2-(2-Furanylmethyl)-5-methylfuran		0.007		
1-(2-Furyl)-2-propanone	0.016	0.003		
2,2'-(1,2-Ethenediyl)*bis*furan	0.020			
5-Methyl-2(5H)-furanone		0.022		0.003
Thiophenoids				
2-Methylthiophene	0.468	0.132	0.423	0.237
3-Methylthiophene	0.078	0.022	0.074	0.039
2, 3-Dimethylthiophene	0.122	0.054	0.106	0.021
2-Acetylthiophene			0.011	0.009
2-Ethylthiophene	0.035	0.002	0.081	0.007
2-Propylthiophene	0.009		0.010	
2-Acetyl-3/5-methylthiophene	0.011	0.005		
Dimethylformylthiophene	0.058	0.017	0.028	0.016
2/5-Methyl-thieno[2,3-b]thiophene	0.016	0.006		
3-Thiophenecarboxaldehyde		0.045		0.023
2-Hydroxymethylthiophene		0.135		0.005
3/5-Methyl-2-thiophenecarboxaldehyde		0.009	0.048	0.082
2-Formyl-3-methylthiophene		0.007	0.028	0.081
2-Formyl-2,3-dihydrothiophene				0.195
2/3-Thiophenemethanol	0.051	0.133	0.044	0.113
Thieno[3,2-b]thiophene	0.148	0.047	0.090	0.051
Dihydrothienothiophene			0.044	0.033
Methyldihydrothienothiophene	0.071	0.015	0.127	0.022
5-Methylthieno[2, 3-d]thiophene	0.125	0.036	0.036	0.007
2-Thiophenethiol	0.157		0.095	
2-Methyl-3-thiophenethiol	0.096	0.026	0.170	0.045
2-Thienylmethanol	0.164	0.065	0.070	0.117
Dihydro-3-(2H)-thiophenone	0.007		0.007	0.008
Dihydro-2-methyl-3(2H)-thiophenone	0.064	0.026	0.161	0.149
5-Methyl-2(5H)-thiophenone	0.014	0.017		
1-(2-Thienyl)-1-propanone			0.015	

Table II. *Continued*

Compounds	Amount (mg / g ribose)			
	RC 5[a]	RCD 5[b]	RC 8.5[c]	RCD 8.5[d]
Furanoids				
Other sulfur-containing compounds				
2-Methyl-3-furanthiol	0.276	0.017	0.075	
bis(2-Methyl-3-furyl)disulfide	0.178	0.009	0.033	
2-Furfurylthiol	0.834	0.321	0.488	0.276
bis(2-Furfuryl)disulfide	0.420	0.205	0.198	0.124
3-Mercapto-2-pentanone	0.124	0.086		
Furfuryl sulfide	0.020	0.012		0.015
2-[(Methylthio)methyl]furan	0.011	0.015		
2-Methyl-3-(methylthio)-butane			0.097	
2, 3-Dihydro-6-methylthieno[2,3c]furan			0.071	0.065
1,2-Dithian-4-one			0.015	
3-Methyl-1,2,4-trithiane	0.004		0.012	0.013
1,3,5-Trithiolane				0.013
3, 5-Dimethyl-1, 2, 4-trithiolane	0.110	0.134	0.223	0.127
Pyrazines				
Pyrazine			0.076	0.101
Methylpyrazine			0.205	0.088
2,5 or 1,6-Dimethylpyrazine				0.021
Ethylpyrazine				0.043
2-(n-Propyl)-pyrazine				0.002
Dihydro-cyclopentapyrazine			0.028	0.043
5H-5-Methyl-6,7-dihydrocyclopentapyrazine			0.010	0.016
Thiazoles				
Thiazole	0.040	0.074	0.160	0.234
Isothiazole	0.020	0.012	0.004	0.003
2-Acetylthiazole			0.067	0.112
5-Ethyl-2, 4-dimethylthiazole	0.035	0.016	0.041	0.016
2, 4, 5-Trimethylthiazole			0.015	0.009
2-Propionylthiazole				0.007

[a] Ribose/cysteine reaction system at pH 5; [b] Ribose/cysteine/DNA reaction system at pH 5; [c] Ribose/cysteine reaction system at pH 8.5; [d] Ribose/cysteine/DNA reaction system at pH 8.5.

inhibited the formation of these thiophenoids with a few exceptions. Compounds such as 2-methylthiophene, 2,3-dimethylthiophene, 2-ethylthiophene, 2-propylthiophene, dihydro-2-methyl-3(2H)-thiophenone and a couple of thienothiophenes all exhibited at least 2 to 3 folds reduction in quantity when DNA was present under both pH conditions. 2-Thiophenethiol and the meaty flavor compound 2-methyl-3-thiophenethiol, which have antioxidant capacity, were also reduced. However, the data also showed that the contents of several

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thiophenes such as thiophenemethanol, 3-thiophenecarboxaldehyde, 2-hydroxymethylthiophene, and 2-formyl-3-methylthiophene were increased as a result of DNA addition. The increase of thiophenemethanol in the presence of DNA is because DNA increases the concentration of furfuryl alcohol, which could react with H_2S to form thiophenemethanol by a single replacement. Thiophenoids are commonly identified from cooked meats with odor threshold in the ppb range. The alkyl thiophenes have aromas reminiscent of roasted onions. So far none of the thiophenes identified in meat appears to be directly responsible for the meaty characteristics (*1*), but may be considered as important contributors to the meaty aroma.

Pyrazine formation in model systems depends largely on pH value (*1,27*). The condensation of amino acid with dicarbonyl compounds known as the Strecker degradation or the condensation between ammonia and dicarbonyls are known possible pathways for pyrazine formation. Since these condensations are favored at higher pH due to the higher availability of unprotonated amino groups in less acidic solutions, pyrazines usually are formed at higher pH environment. In this study, pyrazines were only identified when the pH was increased and the presence of DNA enhanced their formation to a certain extent. One possible explanation is that DNA acted as a nitrogen source necessary for pyrazine formation.

Formation of thiazoles was also pH sensitive. Except that isothiazole was favored at more acidic condition, other thiazoles were either more abundant or only detected when the pH was increased. DNA increased the concentration of thiazole and 2-acetylthiazole but decreased that of 5-ethyl-2,4-dimethylthiazole. Found in roast beef, grilled pork and fried chicken, 5-ethyl-2,4-dimethylthiazole has a nutty, roast, meaty, liver-like flavor and possesses a low odor threshold value of 2 ppb (*1*). Thiazole compounds are usually alkyl substituented and found in roast, grilled or fried products. 2-Acetylthiazole is the only thiazole containing another functional group and commonly detected in meat volatiles (*28*). As indicated by Vernin and Parkanyi (*29*) the most likely route for thiazole formation is the reaction between hydrogen sulfide, ammonia and carbonyl compounds, which are the breakdown products of cysteine and ribose in this system. Addition of DNA into the system possibly increased the availability of ammonia as a result of thermal degradation of DNA.

In order to obtain a better understanding about the effect of DNA on volatile generation in a system of ribose and cysteine, thermal reactions were conducted in several other solutions containing only DNA, or DNA and ribose, or DNA and cysteine. They were heated under the same conditions without pH adjustment, or with pH adjusted to both starting pH 5 and 8.5. The pH of DNA solution was 1.74 before adjustment. Heating this solution generated only a few volatile compounds with a large amount of 4-oxo-pentanoic acid methyl ester, amounting to over 95% of the total area on the GC chromatogram. The other volatiles included cyclohexene and 5-methyl-2(5H)-furanone in small quantities

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(data not shown). However, thermal treatments of the pH adjusted DNA solutions had much less 4-oxo-pentanoic acid methyl ester but more other volatile compounds such as 2-furfuryl alcohol, 2,3-dihydro-1H-inden-1-one, cyclopentanedione, and a couple of *bis*furans (Table III). Being the most abundant in solutions of both pH, 2-furfuryl alcohol appeared to be generated favorably under more basic conditions. Condensation and degradation of 2-furfuryl alcohol likely gave rise to the variety of furan-containing compounds identified. Comparing the volatiles from heating DNA alone to those from heating DNA and ribose together (Table III), one noteworthy point is the furfural formation. Thermal degradation of DNA did not generate identifiable amounts of furfural, but when ribose was added, a large amount was produced. This quantitative difference is possibly due to the fact that DNA contains deoxyribose instead of ribose that is specific to the formation of furfural. In other words, thermal degradation of deoxyribose produces 2-furfuryl alcohol whereas thermal degradation of ribose generates furfural. In the systems containing both ribose and cysteine without DNA, both furfural and furfuryl alcohol can be generated (Table II). In this case, formation of furfuryl alcohol can be derived from reduction of furfural as shown in Figure 1. On the other hand, the formation of furfuryl alcohol from heating DNA other than from furfural conversion suggests a different route, which likely follows hydrolysis of DNA and dehydration of the deoxyribose as illustrated in Figure 2. One interesting compound identified from heating DNA alone or DNA and ribose together is kinetin, which can be derived from the condensation of 2-furfuryl alcohol or furfural with adenine by losing a molecule of H_2O as shown in Figure 3. Its formation appear to be favored under more acidic conditions and facilitated by ribose as shown by the data. Formation of kinetin suggest that DNA bases such as adenine were relatively stable against heat treatment in this experiment. Kinetin is a well-known plant hormone demonstrating a wide variety of biological activities including regulation of gene expression, stimulation of calcium flux, the cell cycle and as an anti-stress and anti-aging compound (*30*). Recent *in vitro* and *in vivo* studies revealing its biological effects made it even more scientifically interesting and commercially attractive as an ingredient for cosmetic products (*30*). Data obtained also indicated that the formation of 2-furfuryl alcohol was preferred under less acidic condition, while furfural formation was favored under more acidic environments, in which ribose reacts with ammonia via 1,2-enolization to form 3-deoxysone that produces furfural via cyclization. Among volatiles listed in Table 3, there were several nitrogen containing compounds including a couple of indoles and pyrroles. 1-(2-Furanylmethyl)-1H-pyrrole was found from heating DNA alone at higher pH. Shown in Figure 1, if pyrrole formation results from the reaction of ammonia with pentose degradation product as proposed by MacLeod and Ames (1988), the formation of 1-(2-furanylmethyl)-1H-pyrrole would indicate that DNA can produce ammonia under thermal conditions, since condensation between 2-

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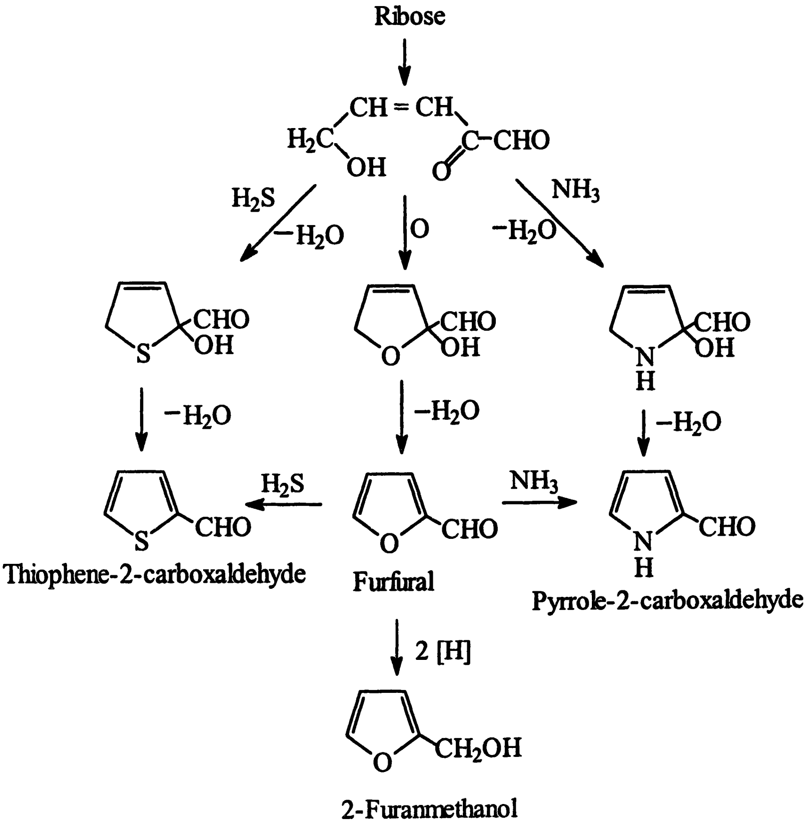
Ribose
H_2S
$-H_2O$
O
NH_3
$-H_2O$
CHO
OH
S
O
N
H
Thiophene-2-carboxaldehyde
Furfural
Pyrrole-2-carboxaldehyde
2 [H]
CH_2OH
2-Furanmethanol

Figure 1. Formation of some related furan, thiophene and pyrrole derivatives from Maillard intermediates (Ref. 6).

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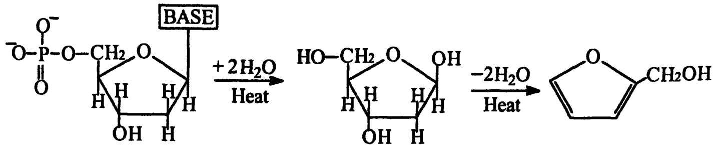
BASE
O⁻
⁻O-P-O-CH₂ O
O
H H H H
OH H
+2H₂O
Heat
HO-CH₂ O OH
H H H H
OH H
-2H₂O
Heat
O
CH₂OH

Figure 2. Formation of 2-furfuryl alcohol from DNA

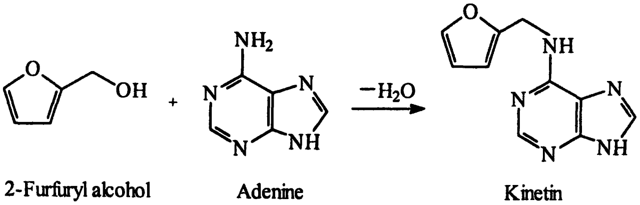
O
OH
+
NH₂
N
N
N
NH
-H₂O
O
NH
N
N
N
NH
2-Furfuryl alcohol
Adenine
Kinetin

Figure 3. Formation of kinetin from 2-furfuryl alcohol and adenine.

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Table III. Volatile compounds generated from the thermal reactions of DNA and between DNA and ribose at both starting pH of 5 and 8.5.

	Amount (mg) (3g DNA)		Amount (mg) (1.5 g Ribose + 3 g DNA)	
Compounds	pH 5	pH 8.5	pH 5	pH 8.5
Furfural			23.396	13.309
2-Furfuryl alcohol	1.245	9.532	1.83	3.223
1-(2-Furanyl)-ethanone	0.080			0.315
5-Methyl-2(5H)-furanone	0.141		0.285	0.075
5-Methyl-2(3H)-furanone	0.090		0.233	
1-(2-Furanyl)-1-propanone				0.012
2-(2-Furanylmethyl)-5-methylfuran				0.038
5-Methyl-2-furancarboxaldehyde	0.006		0.021	0.058
2, 2'-Methylenebisfuran	0.086	0.062	0.299	0.185
2, 2'-[Oxybis(methylene)]*bis*furan	0.008	0.015	0.038	
2, 5-*bis*(2-Furanylmethyl)-furan	0.010			
1-(2-Furanylmethyl)-1H-pyrrole		0.016		
1H-Pyrrole-2-carboxaldehyde				0.026
1,2-Cyclopentanedione	0.180	1.013	0.142	0.494
Pentanoic acid, 4-oxo-methyl ester	0.039	0.012	0.138	0.049
2-Hydroxy-3-methyl-2-cyclopenten-1-one		0.034		0.15
3-Ethyl-2-hydroxy-2-cyclopenten-1-one		0.038		0.092
2-Cyclopentene-1, 4-dione	0.041		0.021	0.026
4-Methoxy-2-cyclopenten-1-one	0.028		0.031	0.023
1, 2-Cyclohexanedione	0.052			0.130
2, 3-Dihydro-1H-inden-1-one	0.351	0.056	0.427	0.387
2 or 3 or 5-Methyl-1H-indole		0.015		
1H-Indole-4-carboxaldehyde	0.007			
Kinetin	0.009		0.031	

furfurylalcohol and pyrrole would give rise to the formation of 1-(2-furanylmethyl)-1H-pyrrole. However, the small amount of these nitrogen-containing compounds suggest that DNA was not a major nitrogen source for providing ammonia.

The reactions between DNA and cysteine generated some well-known sulfur containing compounds, including 2-furfurylthiol, 2-acetyl-3/5-methylthiophene, 2-methyl-thieno[2,3-b]thiophene and two 3, 5-dimethyl-1, 2, 4-trithiolane isomers (Table IV). A large amount of 2-thiophenemethanol was produced likely as the result of a single oxygen atom replacement by sulfur on the furan ring of 2-furfuryl alcohol. Again, furfural was not identified without ribose present in the reaction system. The large amount of 2-furfurylthiol suggest that its formation could arise from condensation not only between

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Table IV. Volatile compounds identified from the reaction between cysteine and DNA

	Amount (mg) (1.212 g Cysteine + 3 g DNA)	
Compounds	pH 5	pH 8.5
Furans		
2-Furfuryl alcohol	2.584	5.064
2, 2'-Methylenebisfuran	0.026	
5-Methyl-2(3H)-furanone	0.018	
2, 5-Dimethylfuran	0.006	
2-Acetyl-5-methylfuran	0.105	
Ketones		
2-Cyclopentene-1, 4-dione	0.006	
Cyclopentanone	0.034	0.077
2-Ethylcyclopentanone	0.007	0.014
2-Methyl-2-cyclopenten-1-one		0.034
3-Methyl-2-cyclopenten-1-one	0.007	0.014
3-Methyl-2-cyclohexen-1-one		0.032
4-(1-Methylethyl)-2-cyclohexen-1-one	0.017	
Thiophenes		
2/3-Methylthiophene	0.064	0.080
Dihydro-3(2H)-thiophenone	0.006	0.021
Dihydro-2-methyl-3(2H)-thiophenone	0.016	0.020
2-Thiophenemethanol	0.652	0.887
2-Acetyl-3-methylthiophene		0.012
2-Acetyl-5-methylthiophene	0.035	0.097
Thieno[3, 2-b]thiophene		0.014
2-Methyl-thieno[2, 3-b]thiophene	0.017	0.117
Pyridines		
2, 6-Dimethylpyridine	0.010	0.009
3-Ethylpyridine		0.026
Thieno[3, 2-c]pyridine		0.255
Pyrroles		
3-Methyl-1H-pyrrole		0.084
2/3-Ethyl-1H-pyrrole		0.038
2-Methyl-4-ethyl-1H-pyrrole	0.027	0.052
3, 4-Diethyl-2-methyl-1H-pyrrole	0.026	
1H-Pyrrole-2-carboxaldehyde		0.450

Continued on next page.

Table IV. *Continued*

Compounds	Amount (mg) (1.212 g Cysteine + 3 g DNA) pH 5	 pH 8.5
Thiazole		
2-Acetylthiazole	0.016	0.132
2-Methylthiazole	0.009	
4, 5-Dihydro-2-methylthiazole	0.009	
5-Ethyl-2-methylthiazole	0.013	
1, 2-Benzisothiazole	0.071	
Others		
Pentanoic acid, 4-oxo-methyl ester	0.023	
2-Furfurylthiol	0.255	0.026
2 or 3 or 5-Methyl-1H-indole		0.020
Furfuryl sulfide	0.013	
3, 5-Dimethyl-1, 2, 4-trithiolane	0.183	0.187
3, 5-Dimethyl-1, 2, 4-trithiolane	0.186	0.157
1, 2, 5-Trithiepane		0.018
1, 2-Ethanedithiol	0.013	

furfural and H_2S but also between furfuryl alcohol and H_2S. This indicates that furfuryl alcohol from DNA could compete with furfural from ribose for H_2S derived from cysteine. Therefore, the increase of thiophenemethanol and the decrease of furfurylthiol resulting from the addition of DNA into the mixture of ribose and cysteine were a result of competition for H_2S. On the other hand, it also suggested it was easier for H_2S to replace the oxygen atom on the furan ring than to react with the carbonyl group of furfural or the hydroxy residue of furfuryl alcohol. In addition, reaction between DNA and cysteine produced several pyrrole-containing compounds, more than those produced by heating DNA alone. Since addition of cycteine could increase the availability of ammonia after thermal degradation, the number and concentration of pyrrole compounds also increased. In this reaction system, no pyrazines were identified. Instead, there were a couple of pyridines produced. This observation further indicats that deoxyribose from DNA is not acting like ribose, which would otherwise follow the Strecker degradation pathway to react with cysteine to produce dicarbonyls and aminoketones for the formation of pyrazine at higher pH. Data in Table III indicated that heating DNA itself, thermal reactions between DNA and ribose did not generate any pyrazine either. These results suggest that formation of pyrazine necessitate the participation of both ribose and cysteine.

In conclusion, the results demonstrated that DNA had complex effects on volatile formation from the Maillard reaction model system. DNA acted as a source to provide deoxyribose for the formation of 2-furfuryl alcohol and other

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degradation products. Deoxyribose and ribose likely follow different pathways to produce mainly 2-furfuryl alcohol and furfural respectively. They compete with each other for ammonia or sulfur precursors available in the system. DNA exerted a quenching effect on the quantities of some heterocyclic sulfur containing compounds such as 2-methyl-3-furanthiol, 2-furfurylthiol and their respective disulfides. Although DNA also exhibited some facilitating ability on the formation of certain nitrogen-containing compounds including pyrazines and thiazoles, the bases that DNA contained were relatively stable and should not be regarded as major nitrogen sources.

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Chapter 31

Isolation of Physiologically Active Compounds from Nutritional Beverages and Vegetables by Countercurrent Chromatography

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The all-liquid chromatographic techniques of high-speed countercurrent chromatography (HSCCC) and multilayer coil countercurrent chromatography (MLCCC) were applied for isolation of flavonol glycosides from tea, shallot and endive, as well as anthocyanins from fruit juices and purple carrot. CCC also allowed the isolation of preparative amounts of the anti-HIV agent chicoric acid from endive. In addition, polymeric pigments from red wine and black tea could be isolated and fractionated by HSCCC and centrifugal precipitation chromatography (CPC). Subsequent LC-MS analyses of separated HSCCC-fractions revealed partial resolution of the complex mixture of tea polyphenols.

In Nutraceutical Beverages; Shahidi, F., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2003.

A rapidly growing number of functional and nutraceutical ingredients are currently finding their way into food and beverages. Despite considerable research activities, especially with regard to natural antioxidants, information on crucial issues remains lacking. For the majority of the putative "healthy" ingredients sound data about bioavailability, bioactivity as well as correct dosing levels are missing, mainly due to multiple reasons. Firstly, standards for most of the key compounds are not available or only offered at a high price which excludes extended biological testing. Hence, synthesis or isolation of the target compounds from natural sources has to be carried out prior to *ex vivo* or *in vivo* studies. By using conventional chromatographic techniques, isolation of preparative amounts of the desired compounds is time-consuming and difficult to achieve since the bioactive constituents are usually present in complex mixtures. In addition, many of the target compounds are delicate to handle because of their polarity and instability. In this chapter, the application of a gentle all-liquid chromatographic technique of countercurrent chromatography (CCC) for isolation of physiologically active compounds from nutritional beverages and vegetables on a preparative scale is reported.

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Experimental

Materials. Endive (*Cichorium endivia* L.) and shallots (*Allium cepa* L. *var. ascalonicum*) were purchased from a local market. An extract from purple carrots (*Daucus carota* L.) was supplied by Dr. Marcus GmbH (Geesthacht, Germany). Black tea was a Darjeeling brand and purchased from a local supplier.

Extraction of Polyphenols from Endive. Endive (2 kg) was chopped and extracted with approximately 2.5 L of methanol/acetic acid (19:1, v/v) for 2 h. The extract was filtered and evaporated *in vacuo*. The residue was neutralized with 2N NaOH and chromatographed on a Polyamide column which was prepared according to a protocol described elsewhere (*6*). The column was eluted with water and methanol. The acidic flavonol glycosides were eluted as a yellow band with methanol/conc. NH_4OH (99.5: 0.5, v/v). The eluate was evaporated *in vacuo* and freeze-dried to yield 580 mg of a brown lyophilisate which was further used for CCC.

Isolation of Anthocyanins from a Commercial Purple Carrot Extract. The extract (30 g) was applied to an Amberlite XAD-7 column (50 cm × 4 cm, Fluka Chemie, Buchs, Switzerland). The column was washed with 1 L of water, in order to remove sugars and organic acids. The anthocyanins were eluted with 500 mL of a mixture of methanol/acetic acid (19:1, v/v). The solvent was concentrated *in vacuo* and freeze-dried to yield ca. 3 g of a dark colored powder which was used for high speed CCC (HSCCC).

Extraction of Flavonol Glycosides from Shallots. French shallots (1 kg) were chopped and extracted with ca. 2 L of methanol for 24 h. The slurry was filtered and the solvent evaporated at 30 °C using a rotary evaporator. The residue was cleaned-up on a XAD-2 column (washing with water and elution of the flavonol glycoside enriched fraction with methanol). The eluate was lyophilized (to yield ca. 4 g) and separated by HSCCC.

Clean-up of Polymeric Pigments (Thearubigins, TR) from Black Tea. A thearubigin fraction was isolated from Darjeeling tea as described elsewhere (*7*). This TR fraction which was free of monomeric compounds was further fractionated using HSCCC (single coil instrument II, see below).

Countercurrent Chromatography (CCC). A high speed Model CCC-1000 (I) manufactured by Pharma-Tech Research Corporation (Baltimore, Maryland) was equipped with 3 preparative coils, connected in series (total volume: 850 mL). The separations were run at a revolution speed of 1000 rpm and at flow rates from 2.5 to 5 mL/min. All samples were dissolved in an 1:1 mixture of light and heavy phase and injected into the system by loop injection. The amount of sample injected varied from 100 mg to 5 g. Stationary phase retention was in the range of 45-80%; 10 mL fractions were collected. Elution was monitored with a Knauer UV-Vis detector. Second CCC system was a Multi-layer Coil Countercurrent Chromatograph (II) by P.C. Inc. (Potomac, Maryland) equipped with a single coil (volume: 360 mL) and a balanced counterweight. Revolution speed was set at 800 rpm, the flow rate was 2.0 mL/min.

HPLC with Diode Array Detection (HPLC-DAD). A Jasco ternary gradient unit LG-980-02, with degasser and MD-910 multiwavelength detector driven by BORWIN chromatography software was used. The chromatographic separation of anthocyanins was performed on a LUNA RP18 column 5 μm (150 mm × 4.6 mm) from Phenomenex (Aschaffenburg, Germany) at ambient temperature. The mobile phase was a linear gradient of water/acetonitrile/conc. formic acid (87:3:10, v/v/v, solvent A) and water/acetonitrile/conc. formic acid (42:50:10, v/v/v, solvent B). Conditions: initial, 100 % A, 0 % B; linear gradient over 35 minutes to 75 % A, 25 % B; returned to the initial conditions; detection at 520 and 320 nm, flow rate: 0.8 mL/min.

Proton Magnetic Resonance Spectroscopy (^{1}H-NMR). All experiments were performed on a Bruker AMX 300 spectrometer (300 MHz). Spectra of anthocyanins were recorded in CD_3OD-CF_3COOD (19:1, v/v). Spectra of flavonol glycosides and other polyphenols were recorded in CD_3OD. *Chicoric acid (dicaffeoyltartaric acid):* ^{1}H-NMR (300 MHz, CD_3OD, ppm): δ 7.65 (2 H, d, J = 16.0 Hz, H 7/7'); 7.08 (2 H, d, J = 2.0 Hz, H 2/2'); 6.99 (2 H, dd, J = 7.5, 2.0 Hz, H 6/6'); 6.80 (2 H, d, J = 7.5 Hz, H 5/5'); 6.36 (2 H, d, J = 16.0 Hz, H8/8'); 5.81 (2 H, s, H2''/H3''). ^{13}C-NMR (75 MHz, CD_3OD, ppm): δ 72.4 (C 2''/C 3''), 113.6 (C 8/C 8'), 115.1 (C 2/C 2'), 116.5 (C 5/C 5'), 123.2 (C 6/C 6'), 127.6 (C 1/C 1'), 146.8 (C 3/C 3'), 148.4 (C 7/C 7'), 149.9 (C 4/C 4'), 167.7 (C 9/C 9'), 169.5 (C 1''/C 4'').

Electrospray Ionization Ion Trap Multiple Mass Spectrometry (ESI-MS/MS). A Bruker Esquire-LC-MS/MS with electrospray ionization in the positive or negative mode was used. As dry gas nitrogen was used. MS/MS-experiments were performed with different fragmentation amplitudes. The set of parameters was optimized for each application and described elsewhere (*7,8*).

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Results and Discussion

"Healthy eating" is in the focus of many food and health professionals. A balanced diet containing fresh fruits and vegetables rich in flavonoids is considered to be beneficial to health. Especially flavonoids which have always been part of the human diet have attracted the interest of numerous research groups. Epidemiological studies show an inverse correlation between intake of flavonoids and occurrence of coronary heart diseases (*9*). In addition to flavonoids (i.e. anthocyanins, catechins, flavonol glycosides), benzoates, cinnamates and lignans are also discussed as agents with potential health benefits.

To test for health benefits many *in vitro* tests are readily available. However, with regard to the bioavailability of these compounds, very little is known so far. For exact quantification of these compounds as well as for further testing of physiological activities, pure reference compounds are required in reasonable amounts.

The CCC is a preparative chromatographic technique that can deliver pure compounds under gentle conditions. Two modern CCC-techniques, i.e. high-speed countercurrent chromatography (HSCCC) and multilayer coil countercurrent chromatography (MLCCC), have been applied for the isolation of bioactive constituents from beverages and vegetables in the present study. A brief overview of the operation principles of both CCC techniques is given below. In multilayer- as well as in high-speed CCC the separating column consists of a Teflon tubing that is wrapped around a holder in several layers (multilayer coil). The coil rotates in a planetary motion around the central axis of a centrifuge. As a consequence, the Archimedean screw force acts as a retainer of the liquid stationary phase in the coil. The mobile phase can be pumped through the coil without displacing the stationary phase. An efficient mixing of the two immiscible phases is achieved since the force field created by the revolution is heterogeneous and results in efficient partitioning of the solutes in the solvent system. The apparatus is designed with an anti-twist mechanism that ensures continuous solvent flow without requiring a rotating seal (*1-5*). As an all-liquid technique, CCC has no solid stationary phase. Therefore, artifact formation as a result of irreversible adsorption effects is minimized. This implies that the technique is especially suited to the preparative isolation of labile natural products.

In the following, experiments dealing with the preparative-scale isolation and development of solvent systems for CCC with focus on flavonoids and other polyphenols from vegetables and nutritional beverages will be reported. The structure of the target compounds are outlined in Figure 1.

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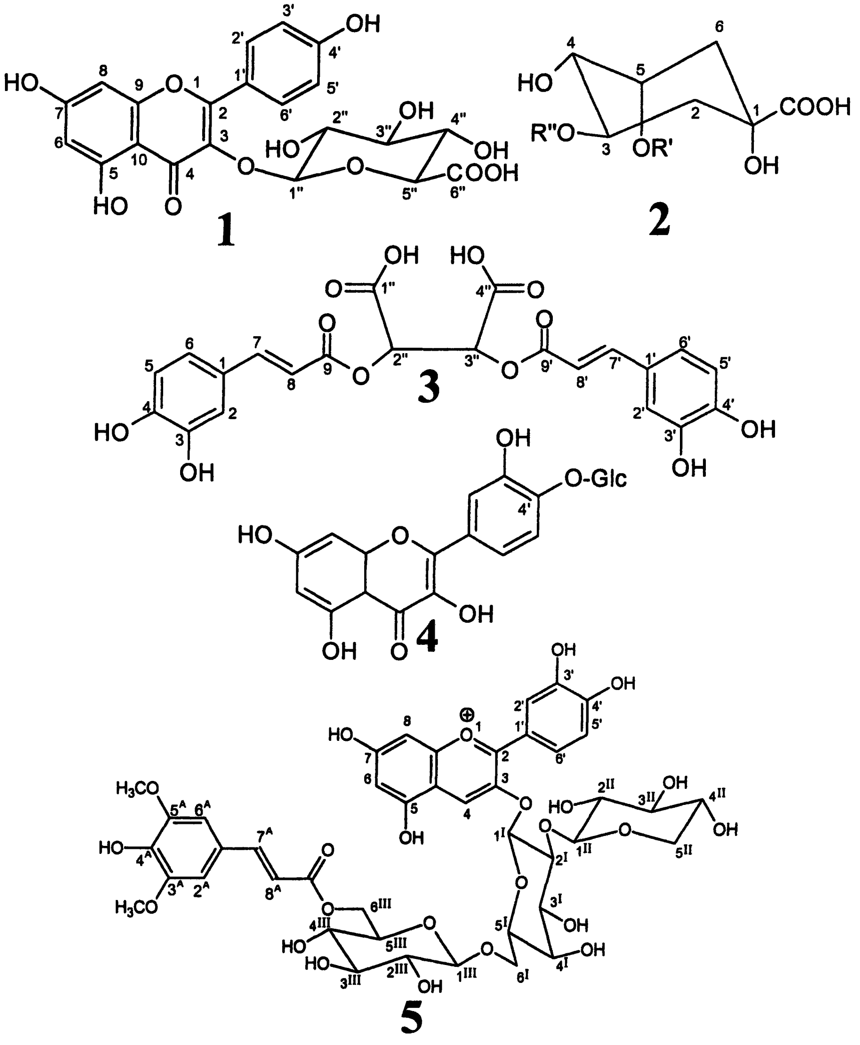

Figure 1. Structures of isolated compounds. 1 Kaempferol-3-O-β-D-glucuronide (K-glcA); 2 3,5-di-caffeoyl-quinic acid (3,5-di-CQA), R', R'' = caffeic acid; 3 chicoric acid (dicaffeoyltartaric acid); 4 Quercetin-4'-O-β-D-glucoside; 5 3-O-{(6-O-sinapoyl)-β-D-glucopyranosyl-(1→6)-[β-D-xylopyranosyl-(1→2)-]β-D-galactopyranosyl} cyanidin (Cy-sin-xyl-glc-gal).

Flavonol Glycosides from Tea, Endive, and Shallots

Tea is a beverage rich in flavonoids and contains about 1-2 % of flavonol-*O*-glycosides besides flavone-*C*-glycosides and flavan-3-ols (catechins). HSCCC proved to be an ideal method for preparative isolation of quercetin-3-*O*-rutinoside and kaempferol-3-*O*-rutinoside. The solvent system used was a mixture of ethyl acetate/water (1:1,v/v). By modifying the separation conditions (solvent system ethyl acetate-*n*-butanol-water 3.5/1.5/5, v/v/v), fractions containing flavonol trisaccharides and flavone-*C*-glycosides could be obtained (*8*).

Endive is widely consumed as salad; two varieties dominate the market, i.e. escariol (var. *latifolium*) and frisée (var. *crispum*). Endive escariol was selected as a source of flavonol glycosides (e.g. kaempferol-3-glucuronide) since its content can be as high as 50 mg/kg (*13*).

The extent of absorption of flavonol glycosides through the gut wall is reported to be dependent on the sugar moiety attached to the aglycone. Hollman et al. (*14-16*) found that flavonoids from onion (quercetin glucosides) were better absorbed than compounds from tea and apple which contain predominantly the rutinoside of quercetin. Hollman and coworkers proposed that the sodium-glucose cotransporter of the small intestine play a role in the absorption of flavonol glucosides. In order to further investigate this hypothesis, additional experiments with another dietary form of flavonols, i.e. the flavonol glucuronides (glcA) were planned. As sources for flavonol glucuronides endive (major compound: kaempferol-3-glcA) and blackberry leaves (major compound: quercetin-3-glcA, results not shown) were chosen (for structure of K-glcA cf. Figure 1).

Figure 2 demonstrates the HSCCC separation of kaempferol-3-glcA **1** from an XAD-2 extract from endive. Three hundred milligrams of the extract yielded 28 mg of **1** in a single run (ESI-MS (negative mode): pseudomolecular ion at *m/z* = 461 $[M-H]^-$, MS/MS of *m/z* = 461: *m/z* 285 [M-H-dehydroglucuronic acid; kaempferol-H]$^-$. The identity of the compound was further proven by ^{1}H- and ^{13}C-NMR spectroscopy. The carboxyl function at C 6'' appeared at 172 ppm in the ^{13}C-NMR spectrum.

As by-products 3,5-di-caffeoyl-quinic acid (3,5-di-CQA) **2** and chicoric acid (dicaffeoyltartaric acid) **3** were isolated in the same run (cf. Figure 2). Compound **2** (yield 7 mg) was identified by comparison of its NMR- and MS spectra (negative mode: molecular peak: 515 $[M-H]^-$, MS/MS of 515: m/z 353 $[M-H-caffeoyl]^-$) with published data (*17*). The identity of chicoric acid (yield 62 mg) was confirmed by ESI-MS (negative mode: molecular peak 473 $[M-H]^-$, MS/MS of 473: m/z 311 $[M-H-caffeoyl]^-$, m/z 293 $[M-H-caffeoyl-H_2O]^-$, m/z

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179 [caffeic acid-H]$^-$) as well as ^{1}H- and ^{13}C-NMR measurements. Chicoric acid was previously isolated from green coffee, chicoree and echinacea and is known to exert anti-HIV as well as antioxidant activity (*18-21*). NMR data was in line with published data (*22*).

Quercetin-4'-*O*-ß-D-glucoside (4) (for structure cf. Figure 1) represents one of the major flavonoids in shallots (*23*). A detailed investigation of the absorption and excretion of onion-derived flavonol glycosides has been reported by Aziz et al. (*24*). The preparative separation of 4 using HSCCC with hexane-ethyl acetate-methanol-water (1/5/1/5; v/v/v/v) is demonstrated in Figure 3. Separation of 4 g of the XAD-2 extract from 1 kg of onions yielded 190 mg of the pure flavonol glycoside 4 (ESI-MS (negative mode): molecular weight 464 Daltons, pseudomolecular ion at m/z 463 [M-H]$^-$; MS/MS of 463: m/z 301 [M-glucosyl-H]$^-$. In some onion species higher amounts of 4 are present which could result in higher yields of flavonol glycoside 4 using this isolation protocol (*25*).

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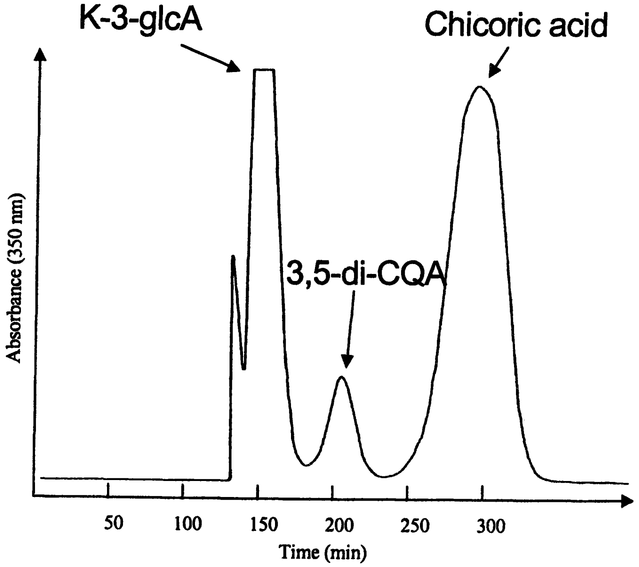

Figure 2. HSCCC separation of polyphenols from endive. K-3-glcA = kaempferol-3-glucuronide (1); 3,5-di-CQA = 3,5-di-caffeoyl-quinic acid (2); chicoric acid = dicaffeoyltartaric acid (3). HSCCC conditions: hexane/ethyl acetate/methanol/water 2/5/2/5, acidified with 0.25 % formic acid; flow rate: 2.8 mL/min.

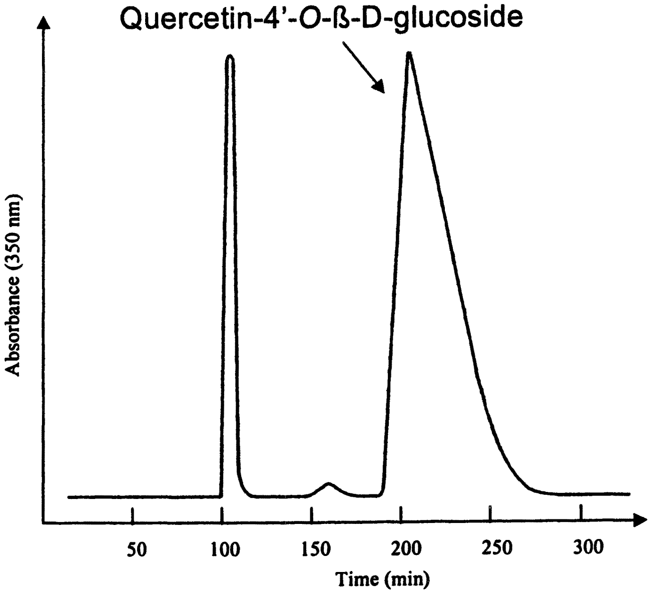

Figure 3. HSCCC separation of XAD-2 extract from onions. For HSCCC conditions see text.

Anthocyanins from Fruit Juices and Purple carrot (*Daucus carota* L.)

Anthocyanins, members of the flavonoid family, are responsible for red, blue and purple colors in many fruits and vegetables. Due to their bioactivity, considerable interest has arisen recently concerning the chemopreventive effects of this class of secondary plant metabolites (*26*).

HSCCC has shown excellent performance in preparative scale separation of the highly polar anthocyanins. Various solvent systems are readily available and the "standard" solvent system for anthocyanin separation, namely TBME/*n*-butanol/acetonitrile/water (2/2/1/5, v/v/v/v, acidified with 0.1 % TFA; less dense layer as stationary phase; flow rate: 5 mL/min) was used for preparative separation of a wide range of anthocyanins (*27*). The separation of delphinidin and cyanidin based glucosides and rutinosides from black currant and other anthocyanins from hibiscus and *Aronia melanocarpa* is described in reference *27*. Other solvent systems for the isolation of anthocyanins with different polarities from grape sources are dealt with in reference *28*.

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In this study anthocyanins from purple carrot (*Daucus carota* L.) were studied. Purple carrots contain both carotenoids and anthocyanins which are located in the outer part of the carrot and therefore, purple carrots are of considerable interest because of their secondary metabolites (*29*). Moreover extracts form purple carrot show sufficient stability for use as natural colorant in various foods due to their acylation with cinnamic acid derivatives (*30*). In order to achieve a better resolution of purple carrot anthocyanins by HSCCC the *n*-butanol content was augmented to the final composition of TBME/*n*-butanol/acetonitrile/water (1/3/1/5, v/v/v/v acidified with 0.1 % TFA). The chromatogram of the HSCCC separation is shown in Fig. 4. The anthocyanins could be identified by comparison with literature data (*10-12*) as derivatives of cyanidin (cf. Table I; Figure 1). All anthocyanins could be obtained in pure form except for compounds **7** and **8** which coeluted and were further separated using preparative HPLC. Separation of 600 mg of an XAD-7 isolate by HSCCC yielded 26 mg of pure Cy-sin-xyl-glc-gal (**5**), 28 mg of Cy-xyl-glc-gal (**6**), 57 mg of Cy-xyl-gal (**7**) in mixture with Cy-fer-xyl-glc-gal (**8**) and 25 mg Cy-pcm-xyl-glc-gal (**9**), respectively. The exact structure of compounds **5** to **9** was adapted from references *10-12* and not verified in this study. The ^{1}H-NMR data are in good agreement with those imported by Baker *et al.* (*31*).

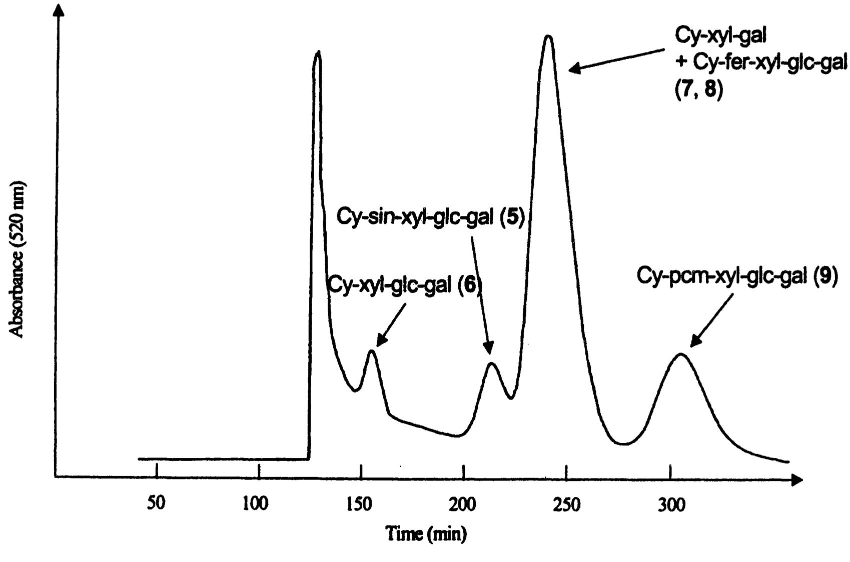

Figure 4. Separation of anthocyanins from purple carrot by HSCCC. Solvent system: TBME/n-butanol/acetonitrile/water (1/3/1/5, acidified with 0.1 % TFA); less dense layer as stationary phase; flow rate: 3.0 mL/min. For abbreviations cf. Table I.

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Table I. Mass spectral data for purple carrot anthocyanins. Abbreviations used: Cy = cyanidin; xyl = xylose; glc = glucose; gal = galactose; sin = sinapic acid; fer = ferulic acid; pcm = para-coumaric acid.

ESI-MS $[M]^+$	ESI-MS/MS $[M]^+$ of aglycone	compound	
743	287	Cy-xyl-glc-gal	(6)
949	287	Cy-sin-xyl-glc-gal	(5)
581	287	Cy-xyl-gal	(7)
919	287	Cy-fer-xyl-glc-gal	(8)
889	287	Cy-pcm-xyl-glc-gal	(9)

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Polymeric pigments from red wine and black tea

Green and black tea are beverages consumed throughout the world and during the past decade, numerous studies have suggested the possible beneficial effects of tea polyphenols in cancer and cardiovascular disease development (*32, 33*). The separation of polymeric pigments from black tea formed by a polyphenol oxidase-mediated reaction from colorless precursors (catechins) during black tea manufacture, often referred to as thearubigins (TR), remains a challenge. Owing to their tremendous complexity, TR elute in the form of a "hump" from RP-HPLC packings. Moreover, it is generally known that TR exert strong affinity to solid supports used in chromatography. Recently, centrifugal precipitation chromatography (CPC), a novel solid support free chromatographic technique, was applied to the fractionation of TR and resulted in a "partial" resolution of the polymeric fraction (*34*). In the case of wine, aging leads to a shift from monomeric compounds to polymerized pigments of unknown structures. CPC also allowed fractionation of polymeric pigments from an aged red wine (*34*).

With respect to the gentle conditions CCC uses and the absence of a solid stationary phase, HSCCC was tested for its ability to fractionate TR. A fraction from tea was isolated using a protocol described by Degenhardt et al. (*7*). This fraction was shown to be free of chromatographically resolved monomeric compounds and was further fractionated using HSCCC (instrument II) using a mixture of two biphasic solvent systems. This allowed adjustment of the polarity of the solvent system in order to achieve the desired partition coefficient. Solvent

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system A consisted of acetonitrile/1 M NaCl (1.5/1, v/v) and system B was ethanol/acetonitrile/saturated $(NH_4)_2SO_4$-solution/water (1/0.5/1.2/1, v/v/v/v). A and B were mixed in the ratio of 1.5 to 1. The separation was both carried out in the head-to-tail elution mode with the less dense organic layer as stationary phase, as well as in the tail-to-head elution mode with the aqueous layer as stationary phase. Six fractions were obtained in both modes (run time 300 min each and 50 min combined in each fraction) and desalted prior to analysis by LC-MS. The pigments were absorbed on a short XAD-7 column, the salt was rinsed with water and elution was carried out with a few mL of methanol.

Figure 5 shows LC-MS analysis of fraction 3 from the head-to-tail separation of 200 mg TR fraction from a Darjeeling black tea. Masses of up to 2100 Daltons were observed in the "partial" resolved hump from TR. Mass spectra at 18.1 and 24.7 min are shown in Figure 6. The mixture is still far too complex to isolate single compounds from the TR and the spectra show a considerable amount of background. However, these results may help to shed some light on the actual size of TR and are in good agreement with results obtained by size exclusion HPLC (*35*). The presented solvent system consisting of a mixture of two different solvent systems is an ideal way to adjust polarity and allows separation of even very hydrophilic polymeric compounds. For a final structure elucidation of TR, in addition to LC-MS, model oxidations as well as NMR analysis of the fractionated TR are being carried out.

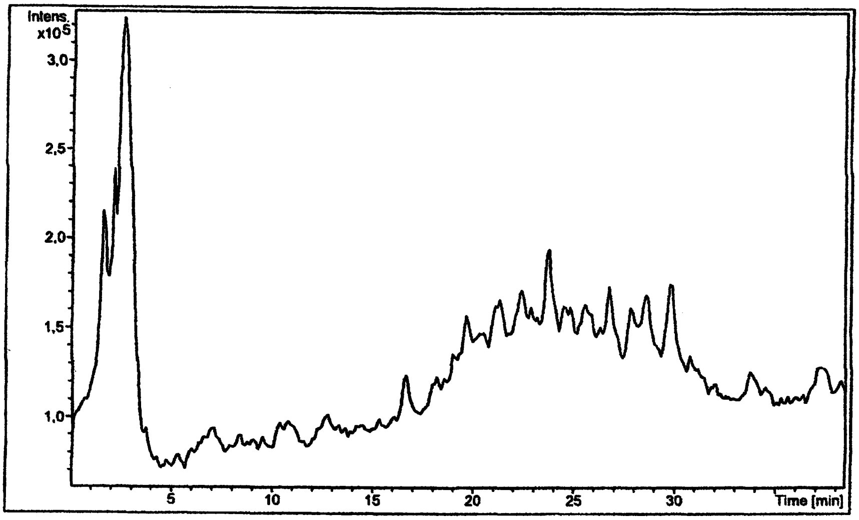

Figure 5. LC-MS analysis of fractionated TR by HSCCC. For conditions see text.

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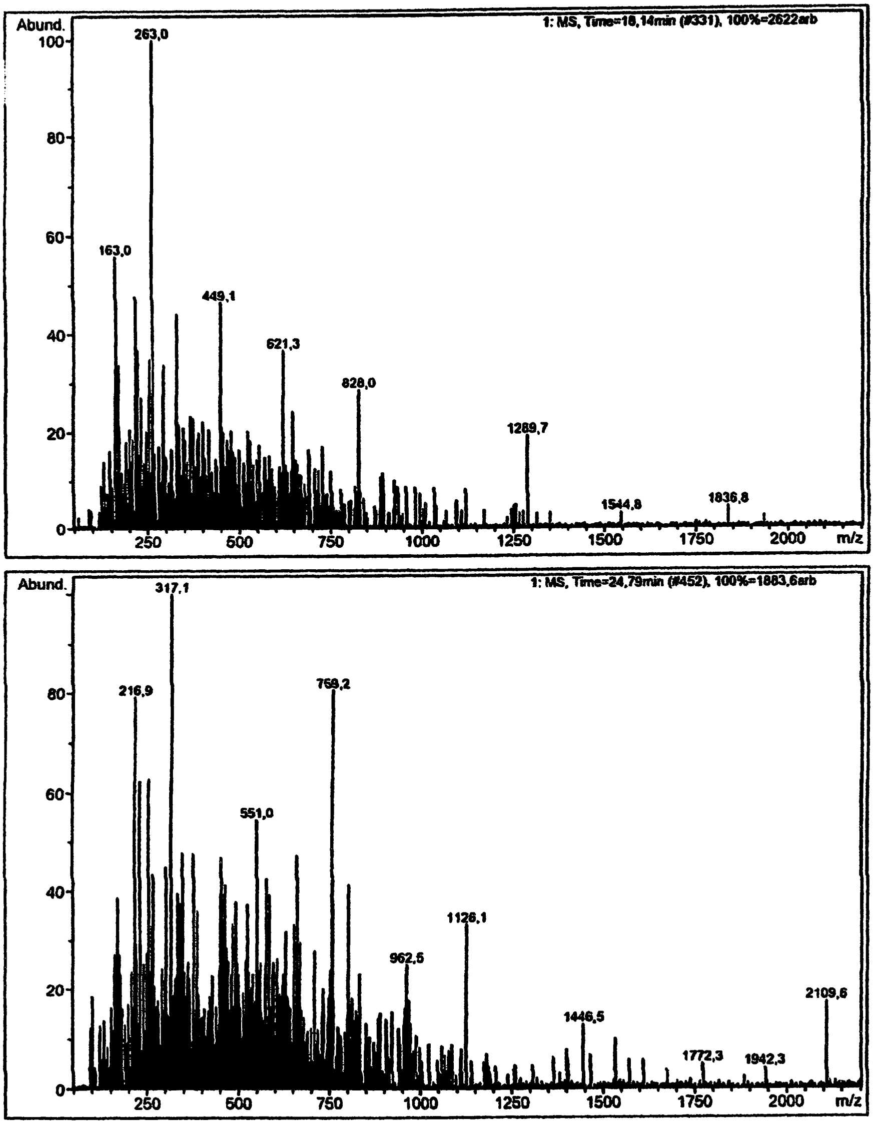

Figure 6. Mass spectra at 18.1 and 24.7 min of the HPLC-MS chromatogram in Figure 5.

Conclusions

The CCC has been successfully applied to the separation of a variety of biologically-active compounds from vegetables and nutritional beverages on a preparative scale. The gentle operation conditions (inert system, room temperature, no solid support) even allow the isolation of labile compounds. Further scale-up of separations presented here is feasible.

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